ADVANCES IN FOOD AND NUTRITION RESEARCH

Volume 45

Steve L. Taylor
ADVISORY BOARD

BRUCE CHASSY
University of Illinois, USA

PATRICK FOX
University College Cork, Republic of Ireland

DENNIS GORDON
North Dakota State University, USA

ROBERT HUTKINS
University of Nebraska, USA

RONALD JACKSON
Quebec, Canada

DARYL B. LUND
University of Wisconsin, USA

CONNIE WEAVER
Purdue University, USA

LOUISE WICKER
University of Georgia, USA

HOWARD ZHANG
Ohio State University, USA

SERIES EDITORS

GEORGE F. STEWART (1948–1982)
EMIL M. MRAK (1948–1987)
STEVE L. TAYLOR (1995– )
ADVANCES IN
FOOD AND NUTRITION
RESEARCH

VOLUME 45

 Edited by

STEVE L. TAYLOR
Department of Food Science and Technology
University of Nebraska
Lincoln, Nebraska
USA

ACADEMIC PRESS
An imprint of Elsevier Science

Amsterdam  Boston  London  New York  Oxford  Paris  San Diego
San Francisco  Singapore  Sydney  Tokyo
CONTENTS

Inositol Phosphates in Foods
Brian Q. Phillippy

I. Introduction .......................................................... 1
II. Chemistry of Inositol Phosphates ............................... 2
III. Metabolism of Inositol Phosphates ......................... 7
IV. Inositol Phosphates in Seeds .................................. 12
V. Inositol Phosphates in Fruits and Vegetables ............... 22
VI. Inositol Phosphates in Animals .............................. 23
VII. Nutritional Importance of Inositol Phosphates ............ 32
VIII. Summary and Conclusions .................................. 41
IX. Future Research Needs ......................................... 42
  Disclaimer .......................................................... 43
  Acknowledgement ............................................... 43
  References ....................................................... 43

Pyrrolizidine Alkaloids in Foods
Roger A. Coulombe, Jr

I. Introduction .......................................................... 61
II. Plant Sources ........................................................ 62
III. Chemical Structures of Pyrrolizidine Alkaloids .......... 65
IV. Pyrrolizidine Alkaloids in Foods and Herbal Medicines .. 66
V. Toxicity of Pyrrolizidine Alkaloids ........................................ 78  
VI. Metabolism of Pyrrolizidine Alkaloids ................................. 81  
VII. Mechanism of Toxic Action .................................................. 83  
VIII. Control of Pyrrolizidine Alkaloids and Future Prospects ........ 91  
Acknowledgements ................................................................. 93  
References .......................................................................... 93  

**Ultrasonic Sensors for the Food Industry**  
John N. Coupland and Raffaella Saggin  

I. Introduction ............................................................................. 102  
II. Theory .................................................................................. 103  
III. Measurement Methods ......................................................... 121  
IV. Applications .......................................................................... 129  
V. Conclusions ........................................................................... 157  
Acknowledgements ................................................................. 158  
References .......................................................................... 159  

**Ozone and Its Current and Future Application in the Food Industry**  
Jin-Gab Kim, Ahmed E. Yousef and Mohammed A. Khadre  

I. Introduction ............................................................................. 168  
II. Ozone Chemistry and Physics: An Overview ......................... 170  
III. Medium for Ozone Treatment ................................................ 178  
IV. Reactor and Equipment Considerations ................................. 182  
V. Application of Ozone in Food Processing ............................... 186  
VI. Selected Food Applications .................................................. 193  
VII. Combination Treatments ...................................................... 200  
VIII. Analytical Methods ............................................................. 204  
IX. Regulatory Status ................................................................. 206  
X. Limitations, Toxicity and Safety ........................................... 206  
Acknowledgements ................................................................. 208  
References .......................................................................... 208
The High Molecular Weight Subunits of Wheat Glutenin and Their Role in Determining Wheat Processing Properties

Peter R. Shewry, Nigel G. Halford, Arthur S. Tatham, Yves Popineau, Domenico Lafiandra and Peter S. Belton

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Introduction</td>
<td>220</td>
</tr>
<tr>
<td>II. The HMW Subunits of Glutenin</td>
<td>224</td>
</tr>
<tr>
<td>III. The Sequences and Structures of HMW Subunits</td>
<td>228</td>
</tr>
<tr>
<td>IV. Experimental Evidence for the Role of HMW Subunits in Dough Mixing and Gluten Viscoelasticity</td>
<td>242</td>
</tr>
<tr>
<td>V. Manipulating HMW Subunit Composition</td>
<td>249</td>
</tr>
<tr>
<td>VI. Experimental Evidence for Differential Effects of Individual HMW Subunits on Mixing and Rheological Properties</td>
<td>256</td>
</tr>
<tr>
<td>VII. The Molecular Basis for Correlations between HMW Subunits and Quality</td>
<td>265</td>
</tr>
<tr>
<td>VIII. Theoretical Basis for Properties of the HMW Subunits and their Role in Gluten Viscoelasticity and Dough Mixing</td>
<td>273</td>
</tr>
<tr>
<td>IX. Conclusions and Future Prospects</td>
<td>289</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>290</td>
</tr>
<tr>
<td>References</td>
<td>291</td>
</tr>
<tr>
<td>INDEX</td>
<td>303</td>
</tr>
</tbody>
</table>
This Page Intentionally Left Blank
CONTRIBUTORS TO VOLUME 45

Numbers in parentheses indicate the page on which the authors’ contributions begin.

Peter S. Belton, School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, UK (219)

Roger A. Coulombe Jr., Graduate Program in Toxicology and Department of Veterinary Sciences, Utah State University, 4620 Old Main Hall, Logan, UT 84332-4620, USA (61)

John N. Coupland, Department of Food Science, Pennsylvania State University, 111 Borland Laboratory, University Park, PA 16802-2504, USA (101)

Nigel G. Halford, IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF, UK (219)

Mohammed A. Khadre, Department of Food Science and Technology, The Ohio State University, 2015 Fyffe Road, Parker Hall, Columbus, OH 43210, USA (167)

Jin-Gab Kim, Department of Food Science and Technology, The Ohio State University, 2015 Fyffe Road, Parker Hall, Columbus, OH 43210, USA (167)

Domenico Lafiandra, Dipartimento di Agrobiologia ed Agrochimica, Università degli Studi della Tuscia, Via San Camillo de Lellis, Viterbo 01100, Italy (219)
Brian Q. Phillippy, United States Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, 1100 Robert E. Lee Boulevard, New Orleans, LA 70124, USA (1)

Yves Popineau, Institut National de la Recherche Agronomique, Centre de Recherches de Nantes, Laboratoire de Biochimie et de Technologie des Protéines, B.P. 71627, Rue de la Géraudière, Nantes 44316, Cedex 03, France (219)

Raffaella Saggin, Department of Food Science, Pennsylvania State University, 111 Borland Laboratory, University Park, PA 16802-2504, USA (101)

Peter R. Shewry, IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF, UK (219)

Arthur S. Tatham, IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF, UK (219)

Ahmed E. Yousef, Department of Food Science and Technology, The Ohio State University, 2015 Fyffe Road, Parker Hall, Columbus, OH 43210, USA (167)
I. INTRODUCTION

Since the middle of the twentieth century, myo-inositol hexakisphosphate, which is commonly known as phytate, has been recognized as an antinutrient for its ability to bind to, precipitate and decrease the bioavailability of divalent and trivalent cationic minerals. Phytate is present in all seeds, usually at
levels between approximately 0.5 and 2% of their dry weight. In diets containing a large proportion of calories derived from grains and/or legumes, an imbalance of phytate and minerals can lead to nutritional deficiencies. Trace minerals such as zinc and iron bind to phytate most tightly and are affected to a greater degree than calcium. A comprehensive review of this subject was published by Reddy et al. (1989) and updated by Zhou and Erdman (1995) and Weaver and Kannan (2002).

Until the 1980s, phytate in foods was almost always quantified using methods that were not specific for inositol hexakisphosphate. During that decade, high-performance liquid chromatography (HPLC) procedures for inositol phosphate analysis opened the way for significant advances in the collection of more accurate data. Recognition that inositol phosphates containing different numbers of phosphate groups were present in appreciable amounts in many foods also created a dilemma as to how the older data should be interpreted. This problem was magnified by the discovery that the different inositol phosphates had different effects on the bioavailability of minerals.

An additional layer of complexity arose at that same time with the ability of some HPLC methods to separate some of the inositol phosphate isomers. Although isomers containing the same number of phosphate groups have not been shown to differ much in their effects on mineral availability, some chemical properties and biological functions have been linked to specific structures. Appreciation of the fundamental importance of inositol phosphates in basic cell physiology coupled with experimental data involving humans and other animals has led to a re-evaluation of the roles of inositol phosphates in food.

In this review the current knowledge about these compounds is compiled and organized in an attempt to provide a context for its interpretation and to create a framework from which scientists can formulate ideas for future research.

II. CHEMISTRY OF INOSITOL PHOSPHATES

A. NOMENCLATURE

There have been several changes in the rules and conventions for naming inositol phosphates since the first myo-inositol monophosphate isomer was identified in soybeans by Ballou and Pizer (1959). This resulted in some confusion in the literature of the following years, when the names assigned to enantiomers became switched and subsequently simplified. The current guidelines were issued in 1989 (NC-IUB, 1989).
Thorough discussions of the naming and numbering of the structures of inositol and inositol phosphates are provided by Posternak (1965) and Cosgrove and Irving (1980). Myo-inositol is one of nine isomers of inositol, which is the common name for cyclohexanehexols. It contains five equatorial and one axial hydroxyl groups numbered from one to six, with the axial hydroxyl designated as position number 2 (I). The molecule is symmetrical on either side of an axis formed by positions 2 and 5. Thus positions 1 and 3 are equivalent, as are positions 4 and 6. The first numbering convention was to depict the hydroxyls as a fraction, arranged with the most numerous hydroxyls above the inositol ring and listed in the numerator and those below the ring in the denominator. Cis hydroxyl groups were assigned the lowest possible numbers. Accordingly, phytate was referred to as myo-inositol 1,2,3,5/4,6-hexakisphosphate (II). Originally inositol phosphate isomers were numbered according to the conventions of carbohydrate chemistry. Optically active isomers were named based on the configuration of D- and L-glyceraldehyde (Lardy, 1954). Phosphates were given the lowest possible numbers and assigned the prefix D when numbered clockwise with the hydroxyl or phosphate at position 1 projecting upward, and L when numbered counterclockwise. Symmetrical isomers, also known as meso compounds, such as inositol 1,3-bisphosphate, had no D or L.

A major reversal of these rules occurred in 1967, when the International Union of Pure and Applied Chemistry and the International Union of Biochemistry decided to change the nomenclature system for cyclitols (IUPAC-IUB, 1968). Following these rules, the positions were still numbered in the direction that would have the lowest possible number for the first phosphate group, but designated as D if the numbers proceeded in a clockwise direction when the hydroxyl or phosphate at position 1 projected downward, or L if the numbers rotated counterclockwise when the hydroxyl or phosphate at position 1 projected downward. The result of this change was that the D and L assignments of all of the inositol
phosphate names in the literature prior to the implementation of these changes became switched. It was also decided at that time to use the Greek prefixes bis, tris, tetrakis, pentakis and hexakis with inositol phosphates to show that the phosphates are singly attached to the inositol carbons. In contrast, pyrophosphate linkages are represented with the Latin prefixes di, tri, tetra, etc., as in nucleotides and inositol polyphosphate pyrophosphates.

An example of these rules is shown below for the enantiomeric pair of D- and L-myo-inositol 1,2,3,4-tetrakisphosphate (III, IV). Pairs of enantiomers are referred to as D and/or L when the proportion of isomers is unequal or unknown, and DL when the isomers are in equal proportion, i.e. a racemic mixture.

Another revision followed the discovery in 1983 that D-myo-inositol 1,4,5-trisphosphate was a second messenger in signaling events that released calcium from intracellular stores to activate various biochemical reactions. In just a few years a complex pathway of inositol phosphate metabolism was uncovered, and hundreds of scientific articles were published. The abbreviation that became widely used at this time was Ins, preceded by a D or L, as needed, and followed by the phosphate positions enclosed in parentheses, and finally a capital P with a subscript to denote the number of phosphates when more than one. In addition, the prefix myo was omitted. Since most of the isomers in these studies had the D configuration, in 1988 the Nomenclature Committee of IUB again decided to modify the rules and name all isomers according to the D orientation and omit the D or L (NC-IUB, 1989). Using the new rules, structures III and IV would be inositol 1,2,3,4-tetrakisphosphate and inositol 1,2,3,6-tetrakisphosphate, or, in abbreviated form, Ins(1,2,3,4)P₄ and Ins(1,2,3,6)P₄, respectively. Inositol phospholipids, or phosphoinositides, are abbreviated similarly, using PtdIns to represent phosphatidylinositol, e.g. PtdIns(4,5)P₂.

B. ANALYSIS

The specificity and accuracy of the analytical methods for phytate and other myo-inositol phosphates has evolved to the point where the means
available to collect information may be greater than the needs of most food and nutrition scientists. The diversity of methods currently in use poses some intricate questions about exactly what data are most desirable and how they should be presented. There is no doubt that the older methods for phytate analysis are not very specific, but some are still used, and, for many food products, provide the only data available in the literature.

Prior to the development of HPLC methods that separate inositol phosphates from one another, most of these compounds usually were measured together to give data intended to represent phytate. These procedures included numerous variations of the ferric chloride precipitation method and the ion exchange method that was approved by the Association of Official Analytical Chemists in 1988. Components of foods such as oxalic acid (McKenzie-Parnell and Guthrie, 1986), gallic acid (Bos et al., 1991), chlorogenic acid (Bos et al., 1991) and polyphosphate compounds that do not contain inositol, such as nucleotides (Phillippy et al., 1988), could also give elevated phytate values. Nevertheless, data obtained from seeds in their native state should be reasonably accurate, since any interfering compounds are likely to be present in small amounts compared to the large quantities of phytate. However, values from foods in which the phytate may have been partially degraded by enzymatic or thermal processes must be viewed with some caution. The low phytate values reported in fruits and vegetables are especially questionable, since those data are the most likely to be significantly inflated due to the presence of nucleotides, oxalate, etc.

HPLC methods specific for phytate first appeared in the early 1980s and were soon expanded to quantify different inositol phosphates present in foods. At about the same time, the burgeoning research in signal transduction revolving around inositol 1,4,5-trisphosphate led to extremely sensitive methods for measuring inositol phosphates in animal cells. The methods developed in these related disciplines have been reviewed (Irvine, 1990; Xu et al., 1992; Skoglund and Sandberg, 2002).

The currently used HPLC methods for the separation of inositol phosphates fall into two basic categories: ion pair and ion exchange. The ion pair procedure developed by Sandberg and Adherinne (1986) has been used the most often by food scientists because it separates inositol tris-, tetrakis-, pentakis- and hexakisphosphates based only on the number of phosphate groups. This simplifies their quantification and provides all the information that is usually wanted. The disadvantages are that isomers are not separated and that other polyphosphates such as nucleotides can interfere (Morris and Hill, 1996). However, nucleotides such as ATP do not appear to be present in sufficient quantities in mature ungerminated
seeds to significantly elevate the data, so this is probably a minor limitation. Some modifications have been suggested to improve the original method (Lehrfeld, 1994). Recently, an inability to use the ion pair method to measure the inositol phosphates in infant cereals was attributed to the combined high mineral and low phytate contents of these foods (Brooks and Lampi, 2001).

The other type of HPLC procedure separates some of the inositol phosphate isomers by ion exchange. The method developed by Phillippy and Bland (1988) separates phytate and some isomers of inositol tris-, tetrakis- and pentakisphosphate. An improved method can now also separate inositol bisphosphates and a few more of the other isomers (Skoglund et al., 1997a, 1998; Carlsson et al., 2001). The ion exchange procedures provide more extensive data than those using ion pairing and are most useful in identifying the specific isomers present in food products or enzymatic reactions. In many cases, however, routine quantification of the numerous individual isomers present in foods may be neither practical nor justified unless the additional information is specifically desired. Whereas the above ion exchange methods employing acidic eluants give better separation of the most highly phosphorylated inositol phosphates, high pH eluants have been found useful in separating those with the lowest numbers of phosphates (Skoglund et al., 1997b, 1998). Ion exchange HPLC can also be used to separate inositol bis- to hexakisphosphates based solely on the number of phosphate groups (Rounds and Nielsen, 1993), but one should beware of the possible interferences from nucleotides such as ADP, which is present in mature soybean seeds in almost a ten-fold excess over ATP (Phillippy et al., 1994). Recently, ion exchange chromatography with high pH eluants and conductivity detection has been used to analyze phytate, other inositol phosphates and inorganic polyphosphates in foods (Sekiguchi et al., 2000; Talamond et al., 2000).

Additional selective methods to analyze inositol phosphates include capillary chromatography and nuclear magnetic resonance (NMR). Several capillary electrophoresis methods have been developed to separate phytic acid and other inositol phosphates, but they do not appear to have been adopted by the scientific community, perhaps because they need further refinement (Skoglund and Sandberg, 2002). NMR methods can be used to simultaneously determine phytic acid and other inositol phosphates in a mixture (Johnson et al., 1995), and $^{31}$P NMR has been used to quantify some of the inositol phosphates in complete and digested feeds (Kemme et al., 1999). The newest evaporative light scattering detectors have the potential to significantly lower the HPLC detection limits for the most highly phosphorylated inositol phosphates in food extracts, provided that the background from interfering compounds is not too high.
III. METABOLISM OF INOSITOL PHOSPHATES

A. SYNTHESIS

The pathway for the synthesis of phytate has not yet been defined with complete certainty. One reason for this is that there are numerous branch points between myo-inositol and phytate, and a single direct pathway may simply be inadequate to portray this complex web of interconnected reactions. Another reason may be that there is more than one possible route, resulting in redundancy to help ensure the production of sufficient phytate to meet the needs of a particular type of cell. In nature different pathways for phytate synthesis appear to have been favored during the evolution of the diverse species of microorganisms and higher life forms. It is believed that all cells of all organisms probably contain some phytate. Studies of the simplest life forms have provided insights into the possible pathways for phytate synthesis in higher plants and animals.

A complete pathway has been reported for the slime mold Dictyostelium (Stephens and Irvine, 1990); the intermediates were identified as Ins(3)P, Ins(3,6)P$_2$, Ins(3,4,6)P$_3$, Ins(1,3,4,6)P$_4$ and Ins(1,3,4,5,6)P$_5$. Each of these isomers was detected in Dictyostelium cells or homogenates incubated with $[^3]$H]inositol, and each of them was converted to InsP$_6$ by Dictyostelium homogenates in separate experiments. Ins(1,2,4,5,6)P$_5$ and Ins(1,2,3,4,6)P$_5$ were also detected in the cells and homogenates and could be phosphorylated to InsP$_6$. However, these pentakisphosphates were probably not the main precursor of InsP$_6$, because they contained lower specific radioactivities than Ins(1,3,4,5,6)P$_5$, and because they, and not the latter, were observed as dephosphorylation products of InsP$_6$. Dictyostelium contains inositol 3-kinase activity, but Ins(3)P may also be derived in part from glucose 6-phosphate or phosphatidylinositol 3-phosphate (Stephens et al., 1990).

Another pathway for InsP$_6$ synthesis in Dictyostelium has been observed, starting with Ins(1,4,5)P$_3$, which is the inositol phosphate second messenger involved in signaling via calcium release. This pathway was located in cell nuclei, and the intermediates were identified as Ins(1,3,4,5)P$_4$ and Ins(1,3,4,5,6)P$_5$ (Van der Kaay et al., 1995). Somewhat similarly, a soluble fraction from the yeast Schizosaccharomyces pombe converts Ins(1,4,5)P$_3$ into Ins(1,3,4,5,6)P$_5$ and InsP$_6$ primarily through Ins(1,4,5,6)P$_4$, but also partially through Ins(1,3,4,5)P$_4$ (Ongusaha et al., 1998).

In animal cells the first pathway identified for InsP$_6$ synthesis begins with the cleavage of Ins(1,4,5)P$_3$ from PtdIns(4,5)P$_2$, followed by the sequential formation of Ins(1,3,4,5)P$_4$, Ins(1,3,4)P$_3$, Ins(1,3,4,6)P$_4$ and Ins(1,3,4,5,6)P$_5$ (Shears, 1989). The latter is by far the predominant InsP$_5$ isomer in animal cells, which also contain the 2-kinase that phosphorylates...
it to form InsP₆ (Ji et al., 1989; Stephens et al., 1991). Evidence from avian erythrocytes suggests that Ins(1,3,4,5,6)P₅ could also be produced from Ins(1)P via the stepwise formation of Ins(1,6)P₂, Ins(1,4,6)P₃, Ins(1,3,4,6)P₄, Ins(3,4,6)P₃ and Ins(3,4,5,6)P₄ (Stephens and Downes, 1990). In addition, a 3-kinase that phosphorylates Ins(1,4,5,6)P₄ as well as Ins(1,2,4,5,6)P₅ has been detected in rat liver (Craxton et al., 1994). Recently, an inositol polyphosphate multikinase that can convert Ins(4,5)P₂ to Ins(1,3,4,5,6)P₅ via Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ was cloned from a rat cDNA library (Saiardi et al., 2001). The enzymes mentioned above and others form a complex network leading to InsP₆ that may provide redundancy to ensure its synthesis and/or regulatory control over the cellular concentrations of its metabolites. Nevertheless, the fundamental route of InsP₆ synthesis in animals is currently unresolved (Irvine and Schell, 2001).

The inositol polyphosphate pathway in plants also appears to have alternate routes to make InsP₆. As in animals, a likely precursor is Ins(1,3,4,5,6)P₅. Kinases that can phosphorylate Ins(1,3,4)P₃ to Ins(1,3,4,5)P₄ and Ins(1,3,4,5,6)P₅ have been identified in Arabidopsis and soybean seeds (Wilson and Majerus, 1997; Phillippy, 1998a). Some evidence has been obtained for the sequential phosphorylation of Ins(3)P, Ins(3,4)P₂, Ins(3,4,6)P₃, Ins(3,4,5,6)P₄, and Ins(1,3,4,5,6)P₅ in Spirodela polyrhiza and Commelina communis (Brearly and Hanke, 1996, 2000). Kinases that phosphorylate several of the inositol pentakisphosphates already containing a phosphate at position 2 have also been observed in mung bean and soybean seeds (Stephens et al., 1991; Phillippy et al., 1994), but those isomers may arise from the degradation of InsP₆ rather than its synthesis. In addition, an Ins(1,4,5)P₃ 6-kinase has been identified in pea roots (Chattaway et al., 1992). Transcripts of the genes for L-Ins(1)P, which is the same as D-Ins(3)P, were observed in the embryo and aleurone layer of developing rice seeds shortly before the appearance of phytate-containing particles called globoids (Yoshida et al., 1999). Furthermore, two maize mutants deficient in InsP₆ synthesis produced reduced amounts of InsP₆, and one had increased levels of other inositol phosphates, though the affected genes have yet to be identified unequivocally (Raboy et al., 2000). One of two types of barley mutants produced less than one fourth as much InsP₆ as the parent line and accumulated 15% of the inositol-bound phosphorus in D and/or L-Ins(1,3,4,5)P₄, hypothetically due to a mutated Ins(1,3,4,5)P₄ 6-kinase gene (Hatzack et al., 2000, 2001).

B. DEGRADATION

Animals, plants and microorganisms all make enzymes that break down phytic acid. Phytases are phosphatases with the ability to use InsP₆ as a
substrate, whereas phytases and other phosphatases can hydrolyze the various InsP\(_6\), InsP\(_5\), InsP\(_4\), InsP\(_3\), InsP\(_2\) and InsP isomers. Monogastric animals including humans lack sufficient phytase in their guts to adequately break down the InsP\(_6\) in diets high in whole grains or legumes. Therefore phytases from plants and microorganisms are sometimes utilized to help degrade the inositol phosphates before and/or after foods are eaten. Inositol phosphates in foods can also be degraded by high temperatures and pressures during thermal processes such as frying and canning. The reduction of phytate content during food processing has been thoroughly reviewed by Reddy et al. (1989) and Sathe and Venkatachalam (2002).

InsP\(_6\) and other inositol phosphates in food may be hydrolyzed by phytases as the food is prepared or while it passes through the gastrointestinal tract. The endogenous phytases of seeds can remove much of the InsP\(_6\) in grains and legumes if they are soaked in aqueous solutions for a number of hours prior to cooking (Larsson and Sandberg, 1992; Gustafsson and Sandberg, 1995; Fredlund et al., 1997; Bergman et al., 1999). The absorption of water initiates the germination of seeds and activates any phytases already present. New enzymes are also synthesized from freshly transcribed RNA. Some ungerminated seeds such as rye contain significant amounts of phytase (Greiner et al., 1998), while others such as maize have very little (Laboure et al., 1993). Fermentation can destroy much of the InsP\(_6\) in foods due to the action of microbial and plant phytases (Sutardi and Buckle, 1985; Gustafsson and Sandberg, 1995; Türk et al., 1996). After food is eaten, any phytases present in the food can break down phytate in the stomach.

The amount of phytase activity in different types of cells and tissues does not appear to be correlated with their InsP\(_6\) content. Ungerminated rye grain contained phytase activity of 3.2 µmol min\(^{-1}\) g\(^{-1}\) at 35°C, and this activity remained fairly constant during 10 days of germination (Greiner et al., 1998). Although ungerminated spelt and barley have negligible phytase activity, after 2 and 4 days, respectively, of germination 1.1 and 1.35 µmol min\(^{-1}\) g\(^{-1}\) of activity at 35°C had accumulated (Koneitzny et al., 1994; Greiner et al., 2000b). Maize roots contained 0.50 µmol min\(^{-1}\) g\(^{-1}\) of phytase activity at 40°C (Hübel and Beck, 1996). In rat small intestine the duodenum, jejunum and ileum contained respectively 6.0, 1.3 and 1.0 µmol min\(^{-1}\) g\(^{-1}\) of activity at 60°C (Rao and Ramakrishnan, 1985), and in rat intestinal mucosal tissue the phytase activity was 0.36 µmol min\(^{-1}\) g\(^{-1}\) at 37°C (Yang et al., 1991a). Vegetables contain phytase activities at levels up to 0.15 µmol min\(^{-1}\) g\(^{-1}\) at 37°C, which is present in green onions (Phillippy, 1998b). The most widely utilized microbial phytases are secreted, although the *Escherichia coli* enzyme is strictly an intracellular protein.
Numerous phytases and other inositol polyphosphate phosphatases have been purified, and some of them have been cloned for experimental and industrial production. The specific activities of some of the purified phytases along with their pH optima are shown in Table I. The phytases with the highest activities in vitro are from *E. coli* and *Peniophora lycii*. Though few of the animal phytases have been purified and studied, their potential contributions to the breakdown of phytate in foods may justify a more thorough exploration of ways to increase their impact.

<table>
<thead>
<tr>
<th>Source</th>
<th>Specific activity (µmol min⁻¹ mg⁻¹)</th>
<th>pH optimum</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice bran</td>
<td>50 (40)</td>
<td>4.4</td>
<td>Hayakawa et al. (1989)</td>
</tr>
<tr>
<td>Maize seedlings</td>
<td>2.3 (55)</td>
<td>4.8</td>
<td>Laboure et al. (1993)</td>
</tr>
<tr>
<td>Spelt seedlings</td>
<td>262 (35)</td>
<td>6.0</td>
<td>Konietzny <em>et al.</em> (1994)</td>
</tr>
<tr>
<td>Maize roots</td>
<td>64 (40)</td>
<td>5.0</td>
<td>Hüb el and Beck, (1996)</td>
</tr>
<tr>
<td>Tomato roots</td>
<td>285 (37)</td>
<td>4.3</td>
<td>Li <em>et al.</em> (1997)</td>
</tr>
<tr>
<td>Rye seeds</td>
<td>517 (35)</td>
<td>6.0</td>
<td>Greiner <em>et al.</em> (1998)</td>
</tr>
<tr>
<td>Scallion leaves</td>
<td>500 (37)</td>
<td>5.5</td>
<td>Phillippy (1998b)</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>260 (37)</td>
<td>6.0</td>
<td>Nakano <em>et al.</em> (1999)</td>
</tr>
<tr>
<td>Oat seedlings</td>
<td>307 (35)</td>
<td>5.0</td>
<td>Greiner and Alminger (1999)</td>
</tr>
<tr>
<td>Barley seedlings</td>
<td>117 (35)</td>
<td>5.0</td>
<td>Greiner <em>et al.</em> (2000b)</td>
</tr>
<tr>
<td>Faba bean seedlings</td>
<td>636 (35)</td>
<td>5.0</td>
<td>Greiner <em>et al.</em> (2001b)</td>
</tr>
<tr>
<td>Rat intestinal mucosa</td>
<td>5.7 (37)</td>
<td>7.5</td>
<td>Yang <em>et al.</em> (1991a)</td>
</tr>
<tr>
<td>Rat liver</td>
<td>0.01 (37)</td>
<td>7.4</td>
<td>Nogimori <em>et al.</em> (1991)</td>
</tr>
<tr>
<td><em>Schwanniomyces castelli</em></td>
<td>441 (70)</td>
<td>4.4</td>
<td>Segueilha <em>et al.</em> (1992)</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>126 (58)</td>
<td>5.5</td>
<td>Ullah and Gibson (1987)</td>
</tr>
<tr>
<td><em>Thermophilus lanuginosus</em></td>
<td>110 (37)</td>
<td>6</td>
<td>Berka <em>et al.</em> (1998)</td>
</tr>
<tr>
<td><em>Peniophora lycii</em></td>
<td>987 (37)</td>
<td>4</td>
<td>Lassen <em>et al.</em> (1998)</td>
</tr>
<tr>
<td><em>Aspergillus terreus</em></td>
<td>142 (37)</td>
<td>6.5</td>
<td>Wyss <em>et al.</em> (1999)</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>26 (37)</td>
<td>6.4</td>
<td>Wyss <em>et al.</em> (1999)</td>
</tr>
<tr>
<td><em>Emericella nidulans</em></td>
<td>29 (37)</td>
<td>6.5</td>
<td>Wyss <em>et al.</em> (1999)</td>
</tr>
<tr>
<td><em>Myceliophthora thermophila</em></td>
<td>42 (37)</td>
<td>5.5</td>
<td>Wyss <em>et al.</em> (1999)</td>
</tr>
<tr>
<td><em>E. coli</em> M15</td>
<td>811 (37)</td>
<td>4.6</td>
<td>Wyss <em>et al.</em> (1999)</td>
</tr>
<tr>
<td><em>E. coli</em> K12</td>
<td>8016 (35)</td>
<td>4.5</td>
<td>Greiner <em>et al.</em> (1993)</td>
</tr>
<tr>
<td><em>Klebsiella terrigena</em></td>
<td>205 (35)</td>
<td>5.0</td>
<td>Greiner <em>et al.</em> (1997)</td>
</tr>
<tr>
<td><em>Bacillus</em> sp. DS11</td>
<td>20 (37)</td>
<td>7.0</td>
<td>Kim <em>et al.</em> (1998)</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>88 (37)</td>
<td>7</td>
<td>Kerovuo <em>et al.</em> (1998)</td>
</tr>
</tbody>
</table>
Phytases are often categorized by their specificity in removing the first phosphate group from InsP$_6$ to form InsP$_5$. To some extent, the initial site of attack appears to be related to the pH optimum of the enzyme. At pH 2.0, which is the lower of its two pH optima, the phytase from the fungus *Aspergillus niger* produces mainly Ins(1,2,4,5,6)P$_5$ (Irving and Cosgrove, 1972). At its higher optimum of pH 5.5 this enzyme still prefers to hydrolyze the 3-phosphate, but forms an increased proportion of Ins(1,2,3,4,5)P$_5$. The phytase from the fungus *P. lycii* appears to form the same products, with increased hydrolysis of the 4- or 6-phosphate at pH 5.5 compared to pH 3.5, but at both pH values Ins(1,2,3,4,5)P$_5$ or Ins(1,2,3,5,6)P$_5$ is the major product (Lassen *et al.*, 1998). NMR was used to identify the products from *P. lycii* phytase but, like HPLC, this technique cannot differentiate between the chemically equivalent 4 and 6 or the 1 and 3 positions. The phytase from the yeast *Saccharomyces cerevisiae* appears to form only Ins(1,2,4,5,6)P$_5$ at pH 4.5 (Greiner *et al.*, 2001a). *E. coli* phytase has an optimum of pH 4.5 and degrades InsP$_6$ via Ins(1,2,3,4,5)P$_5$ (Greiner *et al.*, 2000a). The most studied plant phytase, from wheat bran, has maximal activity at pH 5.2 and produces predominantly Ins(1,2,3,5,6)P$_5$ (Tomlinson and Ballou, 1962). *Paramecium* phytase has a pH 7.0 activity optimum (Freund *et al.*, 1992) and initially forms Ins(1,2,3,4,5)P$_5$ (Van der Kaay and Van Haastert, 1995). Little is known about the plant alkaline phytases such as the lily pollen enzyme, which has an optimum rate at pH 8 and yields Ins(1,2,3,4,6)P$_5$ (Barrientos *et al.*, 1994). The relatively nonspecific multiple inositol polyphosphate phosphatase (MIPP) from rat liver forms similar amounts of (Ins(1,2,4,5,6)P$_5$ and/or Ins(2,3,4,5,6)P$_5$), (Ins(1,2,3,4,5)P$_5$ and/or Ins(1,2,3,5,6)P$_5$), Ins(1,2,3,4,6)P$_5$ and Ins(1,3,4,5,6)P$_5$ from InsP$_6$ at pH 7.4 (Nogimori *et al.*, 1991). However, in the presence of 200 µM Al$^{3+}$, the dominant product from MIPP is Ins(1,2,3,4,6)P$_5$ (Ali *et al.*, 1995).

Following the removal of the first phosphate from InsP$_6$, some phytases and other phosphatases proceed to remove additional phosphates adjacent to a hydroxyl group. Thus the wheat phytase hydrolyzes Ins(1,2,3,5,6)P$_5$ to Ins(1,2,5,6)P$_4$ and Ins(1,2,3,6)P$_4$, followed by the formation of Ins(1,2,6)P$_3$, Ins(1,2,3)P$_3$ and Ins(1,5,6)P$_3$, and so on, until only Ins(1)P, Ins(2)P and possibly myo-inositol remain (Tomlinson and Ballou, 1962; Phillippy, 1989; Nakano *et al.*, 2000). Phytases from other cereal seeds follow a similar sequence, producing Ins(1,2,3,5,6)P$_5$, Ins(1,2,5,6)P$_4$, Ins(1,2,6)P$_3$, Ins(1,2)P$_2$ and Ins(2)P (Greiner and Alminger, 2001). *A. niger* phytase leaves only Ins(2)P, whereas a combination of the phytase and the pH 2.5 optimum acid phosphatase from *A. niger* removes all six phosphates (Wyss *et al.*, 1999). In contrast to wheat phytase, the
predominant isomer formed by *Dictyostelium* phytase from $\text{Ins}(1,2,3,6)\text{P}_4$ is $\text{Ins}(2,3,6)\text{P}_3$ (Adelt *et al.*, 2001). The calcium-dependent phytase from *Bacillus subtilis* appears to cleave alternate rather than adjacent phosphates, resulting in $\text{Ins}(2,4,6)\text{P}_3$ and $\text{Ins}(1,3,5)\text{P}_3$ as the end products (Kerovuo *et al.*, 2000). Inositol phosphates that are intermediates in the synthesis of $\text{InsP}_6$ can be degraded by various phosphatases with characteristic specificities. For example, *Dictyostelium* and the rat liver MIPP can produce $\text{Ins}(1,4,5)\text{P}_3$ from $\text{Ins}(1,3,4,5,6)\text{P}_5$ using either $\text{Ins}(1,3,4,5)\text{P}_4$ or $\text{Ins}(1,4,5,6)\text{P}_4$ as an intermediate (Van Dijken *et al.*, 1995). Additional inositol phosphate phosphatases have been reviewed by Shears (1989).

Thermal degradation of phytate is accelerated by low pH and high pressure. Upon hydrolysis of pure $\text{InsP}_6$ in solution by autoclaving for 1 h at 121°C, the reduction in $\text{InsP}_6$ content at pH 4.0, 7.0 and 10.7 was 81, 64 and 43%, respectively (Phillippy *et al.*, 1987). The inositol phosphate breakdown products from $\text{InsP}_6$ autoclaved at pH 4.0, resembled those formed by *A. niger* phytase, with $\text{Ins}(1,2,4,5,6)\text{P}_5$ and/or $\text{Ins}(2,3,4,5,6)\text{P}_5$ being the predominant $\text{InsP}_5$ isomer, followed by $\text{Ins}(1,2,3,4,5)\text{P}_5$ and/or $\text{Ins}(1,2,3,5,6)\text{P}_5$ (Phillippy and Bland, 1988). At pH 10.8 the hydrolysis showed much less specificity, which resulted in a more even distribution of isomers.

IV. INOSITOL PHOSPHATES IN SEEDS

A. WHOLE RAW SEEDS

The predominant inositol phosphate in whole grains, legumes and nuts is $\text{InsP}_6$, which may account for approximately 0.4–6% of their dry weight (Reddy, 2002). Compared to the $\text{InsP}_6$ content, the other inositol phosphates in raw and dried seeds are present in relatively minor amounts. For this reason, data obtained using some of the nonspecific methods for phytate analysis that measure all polyphosphate compounds, although not 100% accurate, provide estimates of the $\text{InsP}_6$ in these materials. $\text{InsP}_6$ content is partly determined by the phosphate level of the soil, and $\text{InsP}_6$ accumulates mainly during the final stages of soybean seed development (Raboy and Dickinson, 1987). Levels of substances such as nucleotides that interfere in its analysis would not be expected to be influenced by variations in growing conditions to as great an extent as $\text{InsP}_6$. Mechanical processing of dry seeds such as grinding and milling do not result in significant enzymatic or thermal degradation of $\text{InsP}_6$. However, since $\text{InsP}_6$ is often concentrated in specific areas of seeds (Reddy *et al.*, 1989), separation of different anatomical parts may lower or raise the percentage of $\text{InsP}_6$ in the products as compared to the whole seeds. This is especially true for grains such as rice and wheat, where the $\text{InsP}_6$ is found mostly in
the bran and germ. Processing strategies to extract InsP$_6$ from foods derived from seeds have been extensively reviewed (Reddy et al., 1989; Sathe and Venkatachalam, 2002).

The inositol phosphate contents of some of the most widely utilized seeds used as food are listed in Table II. Literature values have been uniformly converted to g/100 g as shown in parentheses because these units are usually used to portray the nutritional composition of foods and they are easy to compare with percentage data, which has traditionally been used to report phytate content. Values of InsP$_6$, InsP$_5$, InsP$_4$ and InsP$_3$ were converted from µmol/g to g/100 g by multiplying by 0.066, 0.058, 0.050 and 0.042, respectively. As mentioned above, InsP$_6$ levels can fluctuate considerably due to environmental and processing effects, and additional variation can result from genetic differences. Therefore the data in Table II represent random examples that may be more or less typical of each kind of seed as it exists throughout the world. InsP$_6$ comprises more than 75% of the inositol phosphates in most of these seeds, and the InsP$_6$ values fall within or near the ranges of the nonspecific “phytate” values reported elsewhere in the literature. InsP$_6$ and InsP$_5$ account for more than 95% of the total inositol phosphates in most raw grains and legumes. InsP$_3$ is undetectable in most of these seeds and InsP$_4$ is usually less than 5% of the total. Therefore, it may not always be necessary to perform routinely quantification of the InsP$_4$ and InsP$_3$ fractions of raw food materials derived from seeds.

Some raw seeds and related products have not been analyzed for the different groups of inositol phosphates, but data have been obtained using HPLC and NMR methods that specifically measured only InsP$_6$. The InsP$_6$ contents of white rice determined by HPLC and polished rice determined by NMR were 0.23 and 0.42%, respectively (O’Neill et al., 1980; Graf and Dintzis, 1982). These values are comparable to the range of 0.14–0.34% obtained by nonspecific methods (Reddy et al., 1982). Although InsP$_6$ accounted for only 55% of the total inositol phosphates in wild rice (Table II), in a nonquantitative study of farm rice the only inositol phosphate detected other than InsP$_6$ was the monophosphate (Asada et al., 1969). Soybean meal was found to contain 1.44% InsP$_6$ by HPLC (Bos et al., 1991), which is similar to the 1.24% InsP$_6$ in raw soybeans, also determined by HPLC (Talamond et al., 2000). Other values for InsP$_6$ obtained by NMR include 0.84–0.97% for 100% extraction wheat flour, 0.13–0.18% for white wheat flour and 0.75% for Chinese millet (O’Neill et al., 1980). Pearl millet and peanuts analyzed by HPLC contained 0.74 and 0.68% InsP$_6$, respectively (Talamond et al., 2000), and sunflower seeds were determined by HPLC to contain 4.48% InsP$_6$ (Cilliers and van Niekert, 1986).
<table>
<thead>
<tr>
<th>Seed</th>
<th>InsP&lt;sub&gt;3&lt;/sub&gt;</th>
<th>InsP&lt;sub&gt;4&lt;/sub&gt;</th>
<th>InsP&lt;sub&gt;5&lt;/sub&gt;</th>
<th>InsP&lt;sub&gt;6&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/g (g/100 g)</td>
<td>µmol/g (g/100 g)</td>
<td>µmol/g (g/100 g)</td>
<td>µmol/g (g/100 g)</td>
</tr>
<tr>
<td>Wheat&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>–</td>
<td>0.52 (0.03)</td>
<td>0.55–3.64 (0.03–0.21)</td>
<td>10.27–16.36 (0.68–1.08)</td>
</tr>
<tr>
<td>Corn&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>0.47–0.95 (0.03–0.05)</td>
<td>14.18–15.91 (0.94–1.05)</td>
</tr>
<tr>
<td>Wild rice&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.67 (0.03)</td>
<td>1.40 (0.07)</td>
<td>3.14 (0.18)</td>
<td>6.36 (0.42)</td>
</tr>
<tr>
<td>Oat&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>–</td>
<td>0.58 (0.03)</td>
<td>0.25–3.16 (0.01–0.18)</td>
<td>9.80–17.25 (0.65–1.14)</td>
</tr>
<tr>
<td>Rye&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>–</td>
<td>0.79 (0.04)</td>
<td>0.68–4.24 (0.04–0.25)</td>
<td>9.31–15.30 (0.61–1.01)</td>
</tr>
<tr>
<td>Barley&lt;sup&gt;abcgf&lt;/sup&gt;</td>
<td>0.05–0.36 (0.00–0.01)</td>
<td>0.12–0.84 (0.01–0.04)</td>
<td>0.44–3.16 (0.03–0.18)</td>
<td>2.94–17.87 (0.19–1.18)</td>
</tr>
<tr>
<td>Sorghum&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>–</td>
<td>0.47–1.55 (0.03–0.09)</td>
<td>7.12–13.98 (0.47–0.92)</td>
<td></td>
</tr>
<tr>
<td>Triticale&lt;sup&gt;a&lt;/sup&gt;</td>
<td>–</td>
<td>0.48 (0.02)</td>
<td>3.12 (0.18)</td>
<td>15.15 (1.00)</td>
</tr>
<tr>
<td>Sesame&lt;sup&gt;a&lt;/sup&gt;</td>
<td>–</td>
<td>1.30 (0.06)</td>
<td>17.81 (1.03)</td>
<td>81.21 (5.36)</td>
</tr>
<tr>
<td>Lupin&lt;sup&gt;g&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>0.00–1.03 (0.00–0.06)</td>
<td>4.76–10.86 (0.31–0.72)</td>
</tr>
<tr>
<td>Pinto beans&lt;sup&gt;adhf&lt;/sup&gt;</td>
<td>–</td>
<td>0.17–2.96 (0.01–0.15)</td>
<td>0.89–8.48 (0.05–0.49)</td>
<td>8.94–14.11 (0.59–0.93)</td>
</tr>
<tr>
<td>Great Northern beans&lt;sup&gt;d&lt;/sup&gt;</td>
<td>–</td>
<td>0.19–0.48 (0.01–0.02)</td>
<td>1.04–2.19 (0.06–0.13)</td>
<td>12.7–17.0 (0.84–1.12)</td>
</tr>
<tr>
<td>Navy beans&lt;sup&gt;dh&lt;/sup&gt;</td>
<td>–</td>
<td>0.14–0.96 (0.01–0.05)</td>
<td>1.24–1.80 (0.07–0.10)</td>
<td>12.4–16.5 (0.82–1.09)</td>
</tr>
<tr>
<td>Baby Lima beans&lt;sup&gt;h&lt;/sup&gt;</td>
<td>–</td>
<td>0.23 (0.01)</td>
<td>2.13 (0.12)</td>
<td>9.96 (0.66)</td>
</tr>
<tr>
<td>Lima beans&lt;sup&gt;d&lt;/sup&gt;</td>
<td>–</td>
<td>0.77 (0.04)</td>
<td>1.50 (0.09)</td>
<td>12.78 (0.84)</td>
</tr>
<tr>
<td>Roman beans&lt;sup&gt;h&lt;/sup&gt;</td>
<td>–</td>
<td>0.02 (0.00)</td>
<td>1.95 (0.11)</td>
<td>10.6 (0.70)</td>
</tr>
<tr>
<td>Red kidney beans&lt;sup&gt;h&lt;/sup&gt;</td>
<td>–</td>
<td>0.16 (0.01)</td>
<td>1.84 (0.11)</td>
<td>13.5 (0.89)</td>
</tr>
<tr>
<td>Red chili beans&lt;sup&gt;h&lt;/sup&gt;</td>
<td>–</td>
<td>0.02 (0.00)</td>
<td>2.18 (0.13)</td>
<td>11.9 (0.78)</td>
</tr>
<tr>
<td>Red (Guerniquesa) beans&lt;sup&gt;s&lt;/sup&gt;</td>
<td>0.21 (0.01)</td>
<td>–</td>
<td>0.33 (0.02)</td>
<td>9.52 (0.63)</td>
</tr>
<tr>
<td>Brown beans&lt;sup&gt;i&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>1.01 (0.06)</td>
<td>12.6 (0.83)</td>
</tr>
<tr>
<td>Black beans&lt;sup&gt;hij&lt;/sup&gt;</td>
<td>–</td>
<td>0.10–0.16 (0.00–0.01)</td>
<td>1.05–1.87 (0.06–0.11)</td>
<td>5.82–15.91 (0.38–1.05)</td>
</tr>
<tr>
<td>Black (Tolosana) beans&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.21 (0.01)</td>
<td>–</td>
<td>0.34 (0.02)</td>
<td>8.55 (0.56)</td>
</tr>
<tr>
<td>Faba beans&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.43 (0.02)</td>
<td>0.26–0.94 (0.01–0.05)</td>
<td>1.29–3.66 (0.07–0.21)</td>
<td>5.23–9.24 (0.34–0.61)</td>
</tr>
<tr>
<td>Soybeans&lt;sup&gt;f&lt;/sup&gt;</td>
<td>–</td>
<td>0.28 (0.01)</td>
<td>1.41 (0.08)</td>
<td>5.79 (0.38)</td>
</tr>
<tr>
<td>Chickpeas (garbanzo beans)&lt;sup&gt;fsh&lt;/sup&gt;</td>
<td>0.62 (0.03)</td>
<td>0.00–0.22 (0.00–0.01)</td>
<td>0.53–1.76 (0.03–0.10)</td>
<td>3.91–6.00 (0.26–0.40)</td>
</tr>
</tbody>
</table>
TABLE II (continued)
INOSITOL PHOSPHATES IN RAW SEEDS

<table>
<thead>
<tr>
<th>Seed</th>
<th>InsP&lt;sub&gt;3&lt;/sub&gt;</th>
<th>InsP&lt;sub&gt;4&lt;/sub&gt;</th>
<th>InsP&lt;sub&gt;5&lt;/sub&gt;</th>
<th>InsP&lt;sub&gt;6&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/g (g/100 g)</td>
<td>µmol/g (g/100 g)</td>
<td>µmol/g (g/100 g)</td>
<td>µmol/g (g/100 g)</td>
</tr>
<tr>
<td>Blackeye peas (cowpeas)&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.01 (0.00)</td>
<td>0.26 (0.01)</td>
<td>2.52 (0.15)</td>
<td>12.6 (0.83)</td>
</tr>
<tr>
<td>Pigeon peas&lt;sup&gt;h&lt;/sup&gt;</td>
<td>–</td>
<td>0.04 (0.00)</td>
<td>2.41 (0.14)</td>
<td>7.96 (0.52)</td>
</tr>
<tr>
<td>Green split peas&lt;sup&gt;ah&lt;/sup&gt;</td>
<td>–</td>
<td>0.17–0.58 (0.01–0.03)</td>
<td>1.36–2.47 (0.08–0.14)</td>
<td>6.06–6.48 (0.40–0.43)</td>
</tr>
<tr>
<td>Yellow split peas&lt;sup&gt;h&lt;/sup&gt;</td>
<td>–</td>
<td>0.12 (0.01)</td>
<td>1.49 (0.09)</td>
<td>8.82 (0.58)</td>
</tr>
<tr>
<td>Lentils&lt;sup&gt;gkh&lt;/sup&gt;</td>
<td>0.32–0.38 (0.01–0.02)</td>
<td>0.21–0.50 (0.01–0.02)</td>
<td>0.83–1.39 (0.05–0.08)</td>
<td>3.70–10.77 (0.24–0.71)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data derived from Lehrfeld (1989).
<sup>b</sup>Data derived from Larsson and Sandberg (1992).
<sup>c</sup>Data derived from Kasim and Edwards (1998).
<sup>d</sup>Data derived from Lehrfeld (1994).
<sup>e</sup>Data derived from Bergman et al. (1999).
<sup>f</sup>Data derived from Trugo et al. (1999).
<sup>g</sup>Data derived from Burbano et al. (1995).
<sup>h</sup>Data derived from Morris and Hill (1996).
<sup>i</sup>Data derived from Gustafsson and Sandberg (1995).
<sup>j</sup>Data derived from Greiner and Konietzny (1998).
<sup>k</sup>Data derived from Kozlowska et al. (1996).
B. PROCESSED FOODS

Processing methods that degrade $\text{InsP}_6$ result in the formation of inositol phosphates containing fewer than six phosphate groups. As the degradation proceeds, the inositol phosphate concentrations individually peak and then fall at rates determined by the processing conditions and the composition of the food. $\text{InsP}_6$ may remain the predominant inositol phosphate even after most of it has been hydrolyzed if the other inositol phosphates accumulate to only low levels due to a slow $\text{InsP}_6$ hydrolysis rate. Intermediates will accumulate when their rate of formation is faster than their rate of degradation. One factor that can limit their rate of formation is that the solubilities of their salts with multivalent minerals are inversely proportional to the number of phosphate groups. The solubilities of the inositol phosphates help to determine their aqueous concentrations and the reaction rates of phytases and other phosphatases during enzymatic hydrolysis. In addition, the rate of hydrolysis depends on the amount of enzyme, and higher concentrations of intermediates transiently accumulate when more enzyme is present.

$\text{InsP}_6$ in foods may be destroyed enzymatically, nonenzymatically or by a combination of these methods. Canning at high temperatures and pressures quickly inactivates phytases and results in nonenzymatic $\text{InsP}_6$ degradation. Similarly, the toasting of breakfast cereals at very high temperatures results in nonenzymatic destruction of $\text{InsP}_6$. Fermentation of breads results in the enzymatic breakdown of much of the $\text{InsP}_6$ due to yeasts and cereal phytases (Harland and Harland, 1980; Larsson and Sandberg, 1991; Türk et al., 1996, 2000). Soaking and hydrothermal processing of legumes and grains prior to cooking also cause much enzymatic loss of $\text{InsP}_6$ (Gustaffson and Sandberg, 1995; Fredlund et al., 1997). Quick soaking for 2 h and overnight soaking for 18 h gave similar levels of $\text{InsP}_3$, $\text{InsP}_4$, $\text{InsP}_5$ and $\text{InsP}_6$ in various cooked beans (Morris and Hill, 1996).

The inositol phosphates in various processed foods are shown in Table III. The data are on a dry weight basis except for some of the flours and breakfast cereals, which are low moisture foods. Compared to the levels in whole raw seeds (Table II), the level of $\text{InsP}_6$ was higher in wheat bran, rice bran and wheat germ but lower in corn bran, wheat flour, corn flour and sorghum flour. $\text{InsP}_6$ was the predominant inositol phosphate in breakfast cereals, cooked beans, peas and lentils, and breads, except for two of the sour breads. There was much greater variation in the $\text{InsP}_6$ values for the different breakfast cereals (0.6–8.82 $\mu$mol/g) and breads (0.17–8.26 $\mu$mol/g) compared to the cooked legumes (4.93–10.1 $\mu$mol/g). The levels of $\text{InsP}_6$ in the cooked legumes were greater than 50% of their levels in the raw seeds.

SOFTbank E-Book Center Tehran, Phone: 66403879,66493070 For Educational Use. www.ebookcenter.ir
<table>
<thead>
<tr>
<th>Food</th>
<th>InsP&lt;sub&gt;3&lt;/sub&gt; µmol/g</th>
<th>InsP&lt;sub&gt;4&lt;/sub&gt; µmol/g</th>
<th>InsP&lt;sub&gt;5&lt;/sub&gt; µmol/g</th>
<th>InsP&lt;sub&gt;6&lt;/sub&gt; µmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g/100 g)</td>
<td>(g/100 g)</td>
<td>(g/100 g)</td>
<td>(g/100 g)</td>
</tr>
<tr>
<td>Wheat bran&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>–</td>
<td>0.8–2.48 (0.04–0.12)</td>
<td>1.0–11.93 (0.06–0.69)</td>
<td>46.9–84.4 (3.09–5.57)</td>
</tr>
<tr>
<td>Corn bran&lt;sup&gt;a&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.8–1.1 (0.05–0.07)</td>
</tr>
<tr>
<td>Rice bran&lt;sup&gt;de&lt;/sup&gt;</td>
<td>–</td>
<td>1.30 (0.06)</td>
<td>9.82–13.36 (0.57–0.77)</td>
<td>99.2–103.5 (6.55–6.83)</td>
</tr>
<tr>
<td>Wheat germ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>–</td>
<td>1.58 (0.08)</td>
<td>9.48 (0.55)</td>
<td>30.1 (1.99)</td>
</tr>
<tr>
<td>Wheat flour, 85% extraction&lt;sup&gt;f&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>0.07 (0.00)</td>
<td>1.24 (0.08)</td>
</tr>
<tr>
<td>Wheat flour, 55% extraction&lt;sup&gt;f&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>0.00–0.02 (0.00)</td>
<td>0.14–0.17 (0.01)</td>
</tr>
<tr>
<td>Corn flour, 95% extraction&lt;sup&gt;e&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>1.33 (0.08)</td>
<td>10.1 (0.67)</td>
</tr>
<tr>
<td>Corn flour, 65% extraction&lt;sup&gt;e&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>0.88 (0.05)</td>
<td>2.14 (0.14)</td>
</tr>
<tr>
<td>Corn flour&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.0 (0.04)</td>
<td>1.9 (0.09)</td>
<td>2.5 (0.14)</td>
<td>1.5 (0.10)</td>
</tr>
<tr>
<td>Oat whole meal flour&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>0.22 (0.01)</td>
<td>10.8 (0.71)</td>
</tr>
<tr>
<td>Rye whole meal flour&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–</td>
<td>0.06 (0.00)</td>
<td>0.50 (0.03)</td>
<td>10.3 (0.68)</td>
</tr>
<tr>
<td>Whole rye meal (1.8–2.0% ash)&lt;sup&gt;h&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>1.4 (0.08)</td>
<td>15.0 (0.99)</td>
</tr>
<tr>
<td>Rye meal (0.5–0.6% ash)&lt;sup&gt;h&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>0.8 (0.05)</td>
<td>7.2 (0.47)</td>
</tr>
<tr>
<td>Sorghum flour&lt;sup&gt;f&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>1.03 (0.06)</td>
<td>5.92 (0.39)</td>
</tr>
<tr>
<td>Quinoa flour&lt;sup&gt;g&lt;/sup&gt;</td>
<td>–</td>
<td>0.1 (0.01)</td>
<td>0.1–0.2 (0.01)</td>
<td>8.6–11.4 (0.57–0.75)</td>
</tr>
<tr>
<td>Soy-based infant formula&lt;sup&gt;l&lt;/sup&gt;</td>
<td>–</td>
<td>0.18 (0.01)</td>
<td>0.95 (0.05)</td>
<td>3.64 (0.24)</td>
</tr>
<tr>
<td>Bran breakfast cereals&lt;sup&gt;k&lt;/sup&gt;</td>
<td>0.4 (0.02)</td>
<td>1.7 (0.08)</td>
<td>4.9 (0.28)</td>
<td>12.0 (0.79)</td>
</tr>
<tr>
<td>Bran flake cereal&lt;sup&gt;j&lt;/sup&gt;</td>
<td>1.41 (0.06)</td>
<td>2.63 (0.13)</td>
<td>6.42 (0.37)</td>
<td>8.82 (0.58)</td>
</tr>
<tr>
<td>Wheat breakfast cereals&lt;sup&gt;k&lt;/sup&gt;</td>
<td>0.7 (0.03)</td>
<td>1.7 (0.08)</td>
<td>3.1 (0.18)</td>
<td>4.9 (0.32)</td>
</tr>
<tr>
<td>Corn breakfast cereals&lt;sup&gt;k&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>0.1 (0.01)</td>
<td>0.6 (0.04)</td>
</tr>
<tr>
<td>Corn flakes cereals&lt;sup&gt;k&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>0.52 (0.03)</td>
<td>1.04 (0.07)</td>
</tr>
<tr>
<td>Rice breakfast cereals&lt;sup&gt;k&lt;/sup&gt;</td>
<td>0.1 (0.00)</td>
<td>0.2 (0.01)</td>
<td>0.6 (0.03)</td>
<td>0.9 (0.06)</td>
</tr>
<tr>
<td>Oat breakfast cereals&lt;sup&gt;k&lt;/sup&gt;</td>
<td>0.2 (0.01)</td>
<td>1.2 (0.06)</td>
<td>3.3 (0.19)</td>
<td>7.6 (0.50)</td>
</tr>
<tr>
<td>Mixed grain breakfast cereals&lt;sup&gt;k&lt;/sup&gt;</td>
<td>0.1 (0.00)</td>
<td>0.5 (0.02)</td>
<td>1.6 (0.09)</td>
<td>3.3 (0.22)</td>
</tr>
<tr>
<td>Müsli cereals&lt;sup&gt;h&lt;/sup&gt;</td>
<td>–</td>
<td>1.5 (0.07)</td>
<td>2.6 (0.15)</td>
<td>8.3 (0.55)</td>
</tr>
<tr>
<td>White wheat bread&lt;sup&gt;ov&lt;/sup&gt;</td>
<td>–</td>
<td>0.17 (0.01)</td>
<td>0.25–0.34 (0.01–0.02)</td>
<td>0.17–0.93 (0.01–0.06)</td>
</tr>
</tbody>
</table>
### TABLE III (continued)

INOSITOL PHOSPHATES IN PROCESSED FOODS

<table>
<thead>
<tr>
<th>Food</th>
<th>(\text{InsP}_3) µmol/g</th>
<th>(\text{InsP}_4) µmol/g</th>
<th>(\text{InsP}_5) µmol/g</th>
<th>(\text{InsP}_6) µmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>French bread(^h)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.75 (0.05)</td>
</tr>
<tr>
<td>Wheat and oat bread(^h)</td>
<td>3.13 (0.13)</td>
<td>1.90 (0.10)</td>
<td>2.56 (0.15)</td>
<td>8.26 (0.54)</td>
</tr>
<tr>
<td>Wheat and rye bread(^h)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2.62 (0.17)</td>
</tr>
<tr>
<td>Sour rye and wheat bread(^h)</td>
<td>3.02 (0.13)</td>
<td>–</td>
<td>0.60 (0.03)</td>
<td>0.60 (0.04)</td>
</tr>
<tr>
<td>Sour wheat and potato bread(^h)</td>
<td>1.43 (0.06)</td>
<td>1.59 (0.08)</td>
<td>–</td>
<td>0.48 (0.03)</td>
</tr>
<tr>
<td>Sour buckwheat bread(^h)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.61 (0.04)</td>
</tr>
<tr>
<td>Crispbread(^h)</td>
<td>–</td>
<td>1.00 (0.05)</td>
<td>1.80 (0.10)</td>
<td>5.90 (0.39)</td>
</tr>
<tr>
<td>Whole wheat pasta(^h)</td>
<td>–</td>
<td>–</td>
<td>1.50 (0.09)</td>
<td>13.50 (0.89)</td>
</tr>
<tr>
<td>Regular pastas(^h)</td>
<td>–</td>
<td>1.10 (0.05)</td>
<td>0.80 (0.05)</td>
<td>6.50 (0.43)</td>
</tr>
<tr>
<td>Four cereals pasta(^h)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5.40 (0.36)</td>
</tr>
<tr>
<td>Wheatmeal porridge flakes, raw(^h)</td>
<td>–</td>
<td>–</td>
<td>2.50 (0.14)</td>
<td>13.90 (0.92)</td>
</tr>
<tr>
<td>Ricemeal porridge flakes, raw(^h)</td>
<td>–</td>
<td>–</td>
<td>0.40 (0.02)</td>
<td>2.70 (0.18)</td>
</tr>
<tr>
<td>Oatmeal porridge flakes, raw(^h)</td>
<td>–</td>
<td>1.30 (0.06)</td>
<td>4.00 (0.23)</td>
<td>14.10 (0.93)</td>
</tr>
<tr>
<td>Ryemeal porridge flakes, raw(^h)</td>
<td>–</td>
<td>–</td>
<td>2.30 (0.13)</td>
<td>14.00 (0.92)</td>
</tr>
<tr>
<td>Barleymeal porridge flakes, raw(^h)</td>
<td>–</td>
<td>–</td>
<td>0.70 (0.04)</td>
<td>4.20 (0.28)</td>
</tr>
<tr>
<td>Pinto beans, cooked(^l)</td>
<td>0.20 (0.01)</td>
<td>0.91 (0.04)</td>
<td>3.33 (0.19)</td>
<td>8.14 (0.54)</td>
</tr>
<tr>
<td>Great Northern beans, cooked(^l)</td>
<td>0.23 (0.01)</td>
<td>1.05 (0.05)</td>
<td>3.60 (0.21)</td>
<td>9.24 (0.61)</td>
</tr>
<tr>
<td>Navy beans, cooked(^l)</td>
<td>0.13 (0.00)</td>
<td>0.68 (0.03)</td>
<td>3.07 (0.18)</td>
<td>8.80 (0.58)</td>
</tr>
<tr>
<td>Baby Lima beans, cooked(^l)</td>
<td>0.25 (0.01)</td>
<td>1.08 (0.05)</td>
<td>3.07 (0.18)</td>
<td>7.08 (0.47)</td>
</tr>
<tr>
<td>Roman beans, cooked(^l)</td>
<td>0.08 (0.00)</td>
<td>0.73 (0.04)</td>
<td>3.25 (0.19)</td>
<td>9.17 (0.60)</td>
</tr>
<tr>
<td>Red kidney beans, cooked(^l)</td>
<td>0.19 (0.01)</td>
<td>1.02 (0.05)</td>
<td>2.81 (0.16)</td>
<td>9.12 (0.60)</td>
</tr>
<tr>
<td>Red kidney beans, canned(^l)</td>
<td>0.45 (0.02)</td>
<td>0.91 (0.04)</td>
<td>3.19 (0.18)</td>
<td>7.51 (0.50)</td>
</tr>
<tr>
<td>Red chili beans, cooked(^l)</td>
<td>0.07 (0.00)</td>
<td>0.81 (0.04)</td>
<td>3.37 (0.19)</td>
<td>10.1 (0.67)</td>
</tr>
<tr>
<td>Black beans, cooked(^l)</td>
<td>0.18 (0.01)</td>
<td>0.98 (0.05)</td>
<td>3.62 (0.21)</td>
<td>9.96 (0.66)</td>
</tr>
<tr>
<td>Chickpeas, cooked(^l)</td>
<td>0.10 (0.00)</td>
<td>0.56 (0.03)</td>
<td>2.04 (0.13)</td>
<td>5.18 (0.34)</td>
</tr>
<tr>
<td>Blackeye peas, cooked(^l)</td>
<td>0.22 (0.01)</td>
<td>0.89 (0.04)</td>
<td>3.38 (0.20)</td>
<td>9.67 (0.64)</td>
</tr>
<tr>
<td>Pigeon peas, cooked(^l)</td>
<td>0.22 (0.01)</td>
<td>0.96 (0.05)</td>
<td>2.77 (0.16)</td>
<td>5.97 (0.39)</td>
</tr>
</tbody>
</table>
## TABLE III (continued)

<table>
<thead>
<tr>
<th>Food</th>
<th>InsP$_3$ $\mu$mol/g</th>
<th>(g/100 g)</th>
<th>InsP$_4$ $\mu$mol/g</th>
<th>(g/100 g)</th>
<th>InsP$_5$ $\mu$mol/g</th>
<th>(g/100 g)</th>
<th>InsP$_6$ $\mu$mol/g</th>
<th>(g/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green split peas, cooked$^l$</td>
<td>0.07 (0.00)</td>
<td>0.45 (0.02)</td>
<td>1.73 (0.10)</td>
<td>4.93 (0.32)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yellow split peas, cooked$^l$</td>
<td>0.05 (0.00)</td>
<td>0.52 (0.03)</td>
<td>1.53 (0.09)</td>
<td>7.35 (0.48)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lentils, cooked$^l$</td>
<td>0.44 (0.02)</td>
<td>0.97 (0.05)</td>
<td>3.62 (0.21)</td>
<td>7.09 (0.47)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bean sprouts, canned$^l$</td>
<td>0.20 (0.01)</td>
<td>0.22 (0.01)</td>
<td>0.60 (0.03)</td>
<td>1.57 (0.10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tofu$^j$</td>
<td>–</td>
<td>–</td>
<td>1.21 (0.07)</td>
<td>22.0 (1.45)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn dough$^g$</td>
<td>–</td>
<td>–</td>
<td>3.31 (0.19)</td>
<td>2.24 (0.15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cassava dough$^g$</td>
<td>–</td>
<td>–</td>
<td>0.34 (0.02)</td>
<td>0.21 (0.01)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Banku$^g$</td>
<td>–</td>
<td>–</td>
<td>2.34 (0.14)</td>
<td>2.12 (0.14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ekuegbemi$^g$</td>
<td>–</td>
<td>–</td>
<td>1.03 (0.06)</td>
<td>1.52 (0.10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fufu$^g$</td>
<td>–</td>
<td>–</td>
<td>0.60 (0.03)</td>
<td>0.98 (0.06)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gari$^g$</td>
<td>–</td>
<td>–</td>
<td>0.17 (0.01)</td>
<td>0.59 (0.04)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fanti kenkey$^g$</td>
<td>–</td>
<td>–</td>
<td>2.26 (0.13)</td>
<td>1.15 (0.08)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ga kenkey$^g$</td>
<td>–</td>
<td>–</td>
<td>2.05 (0.12)</td>
<td>1.18 (0.08)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat-based meal, cooked$^m$</td>
<td>2.79 (0.12)</td>
<td>2.98 (0.15)</td>
<td>4.53 (0.26)</td>
<td>12.3 (0.81)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rice-based meal, cooked$^m$</td>
<td>1.62 (0.07)</td>
<td>1.98 (0.10)</td>
<td>2.53 (0.15)</td>
<td>5.32 (0.35)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maize-based meal, cooked$^m$</td>
<td>1.86 (0.08)</td>
<td>1.42 (0.07)</td>
<td>2.84 (0.16)</td>
<td>7.73 (0.51)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearl millet-based meal, cooked$^m$</td>
<td>2.29 (0.10)</td>
<td>2.74 (0.14)</td>
<td>4.40 (0.25)</td>
<td>11.0 (0.72)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Finger millet-based meal, cooked$^m$</td>
<td>2.17 (0.09)</td>
<td>1.68 (0.08)</td>
<td>3.50 (0.20)</td>
<td>10.9 (0.72)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sorghum-based meal, cooked$^m$</td>
<td>2.21 (0.09)</td>
<td>2.30 (0.11)</td>
<td>3.66 (0.21)</td>
<td>9.82 (0.65)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Data derived from Lehrfeld (1989).
$^b$Data derived from Sandberg and Ahderinne (1986).
$^c$Data derived from Sandberg and Svanberg (1991).
$^e$Data derived from Lehrfeld (1994).
$^f$Data derived from Brune et al. (1992).
$^g$Data derived from Ferguson et al. (1993).
$^h$Data derived from Plaami and Kumpulainen (1995).
$^i$Data derived from Valencia et al. (1999).
$^j$Data derived from Phillippy et al. (1988).
$^k$Data derived from Morris and Hill (1995).
$^l$Data derived from Morris and Hill (1996).
$^m$Data derived from Agte et al. (1999).
Brune et al. (1992) studied the breakdown of InsP$_6$ during breadmaking. Wheat flour of 85% extraction contained 0.829 and 0.42 g/kg of InsP$_6$ and InsP$_5$, respectively. Bread made from this flour had 0.049, 0.008, 0.011 and 0.040 g/kg InsP$_6$, InsP$_5$, InsP$_4$ and InsP$_3$, respectively, based on the dry weight of the flour used. In contrast, wheat flour of 55% extraction contained 0.089–0.106 and 0.005–0.006 g/kg InsP$_6$ and InsP$_5$, respectively. The levels of InsP$_6$, InsP$_5$, InsP$_4$ and InsP$_3$ in bread made from this flour were 0.003–0.013, 0.001–0.005, 0.001–0.002 and 0.005–0.020 g/kg, respectively. The loss of InsP$_6$ during breadmaking was 94% for the 85% extraction flour.

The effect of pH on the hydrolysis of InsP$_6$ in wheat breads containing varying amounts of rye bran, oat flour or oat bran was investigated by Larsson and Sandberg (1991). The acidities of the doughs were raised by the addition of sour dough or lactic acid to pH 3.9–5.5, which resulted in breads with pH 4.2–5.7. The sum of InsP$_6$ and InsP$_5$ was reduced by 79–97% in the breads containing sour dough, whereas the total of InsP$_6$ plus InsP$_5$ decreased 44–97% in breads with lactic acid. Optimum hydrolysis occurred when the pH was between 4.3 and 5.1 in both the dough and the bread. Less InsP$_6$ plus InsP$_5$ was destroyed in breads containing oat bran than in the breads made with rye bran or oat flour owing to lower phytase activities in the oat bran.

In breads made from equal parts of whole and white wheat flours, the addition of lactic acid and A. niger phytase completely eliminated InsP$_6$ and InsP$_5$ (Türk and Sandberg, 1992). Results in the same study also showed that unfermented milk inhibited the destruction of InsP$_6$ and InsP$_5$ to a greater extent than could be accounted for by its calcium content. Türk et al. (1996) investigated the isomeric structures of the inositol phosphates present in whole wheat bread. Ion chromatography revealed that D- and/or L-Ins(1,2,3,4,5)P$_5$ was the predominant InsP$_5$, followed by D- and/or L-Ins(1,2,4,5,6)P$_5$, Ins(1,2,3,4,6)P$_5$ and Ins(1,3,4,5,6)P$_5$. Similar amounts of what appeared to be D- and/or L-Ins(1,2,3,4)P$_4$ and D- and/or L-Ins(1,2,5,6)P$_4$ were also present, most likely due to the action of the wheat and yeast phytases.

Kozlowska et al. (1996) ground raw lentils containing 10.77, 1.07, 0.50 and 0.37 µmol/g InsP$_6$, InsP$_5$, InsP$_4$ and InsP$_3$, respectively, into flour which was then fermented for 4 days. Suspensions of 79 g lentil flour per liter of sterile tap water fermented at 28 or 42°C lost 73 and 78%, respectively, of their InsP$_6$. In contrast, suspensions of 221 g flour per liter fermented similarly had InsP$_6$ decreases of only 63 and 68% at 28 and 42°C, respectively. Interestingly, InsP$_3$ appeared to increase to fairly high levels, 1.51 and 1.91 µmol/g, respectively, in the latter two suspensions after 4 days. However, additional work is needed to determine the
composition of these fractions, since nucleotides such as ATP will elute along with InsP\(_3\) in the ion pair HPLC analysis (Morris and Hill, 1996).

Optimized procedures have been developed to degrade the inositol phosphates in pea flour (Fredrikson \textit{et al}., 2001b). At pH 7.5 and 45°C, 66% of the sum of InsP\(_6\), InsP\(_5\), InsP\(_4\) and InsP\(_3\) was eliminated by endogenous phytase activity within 10 h. The predominant InsP\(_5\) and InsP\(_4\) breakdown products were Ins(1,2,3,4,6)P\(_5\) and D- and/or L-Ins(1,2,3,4)P\(_4\), which is a pattern similar to that of the alkaline phytase of lily pollen (Barrientos \textit{et al}., 1994). A modified process employing an exogenous microbial phytase for just 1 h was used to prepare a dephytinized pea protein isolate flour intended for test production of infant formulas (Fredrikson \textit{et al}., 2001a).

To increase the reduction of InsP\(_6\) during the soaking of black beans containing 15.91, 1.72, 0.16 and 0 µmol/g of InsP\(_6\), InsP\(_5\), InsP\(_4\) and InsP\(_3\), respectively, the effects of pH, temperature and exogenous enzymes were evaluated (Greiner and Konietzny, 1999). When the pH of the soaking buffer was adjusted to pH values from 4.5 to 8.0, the greatest degradation of InsP\(_6\) occurred at pH 6.0 during a 15 h incubation at 50°C and resulted in 7.64, 1.40, 3.85 and 4.75 µmol/g of InsP\(_6\), InsP\(_5\), InsP\(_4\) and InsP\(_3\), respectively. As mentioned above for lentils, large apparent InsP\(_3\) values could conceivably be due to nucleotides such as ATP. Whereas soaking overnight in water at room temperature followed by cooking resulted in only an 8% decrease in the sum of InsP\(_6\) and InsP\(_5\), soaking at pH 6.0 and 60°C for 15 h led to a 54% reduction after cooking. Addition of \textit{E. coli} or rye phytase to the soaking buffer, 50 mM sodium acetate pH 5.5, during the last 2 h of a 15 h soak, had no effect at 25°C, but reduced the sum of InsP\(_6\) and InsP\(_5\) by 29 and 34%, respectively, at 50°C. Cooking led to decreases in beans soaked with \textit{E. coli} or rye phytases at both temperatures such that the sums of InsP\(_6\) and InsP\(_5\) decreased by 46 and 39%, respectively, in beans soaked at 25°C and by 82 and 70%, respectively, in beans soaked at 50°C. Germination of black beans at 25°C in the dark required 14 days to achieve a 47% reduction in the sum of InsP\(_6\) and InsP\(_5\).

Grains can also be soaked during their initial treatment in procedures such as steeping, malting or hydrothermal processing. Soaking leads to the breakdown of InsP\(_6\) by endogenous phytases in wheat, rye, oat, barley and corn (Sandberg and Svanberg, 1991; Larsson and Sandberg, 1992; Hull and Montgomery, 1995; Fredlund \textit{et al}., 1997). Bergman \textit{et al}.
(1999) optimized the hydrothermal processing of barley to degrade InsP\(_6\) and increase the level of myo-inositol. Variables were the temperature \((T_1)\) of the first 1 h wet and 5 h dry steeps, the temperature \((T_2)\) of the second 1 h wet and 15 h dry steeps, and the concentration and volume of lactic
acid in the wet steeps. InsP$_6$ was lowered by 96% to a final concentration of 0.5 µmol per g dry weight using 48 and 50°C for $T_1$ and $T_2$, respectively, and 3.2 volumes of 0.8% lactic acid. Myo-inositol was increased from 0.56 to 2.68 µmol per g dry weight with 48°C for $T_1$, during which the dry steep was prolonged to 21 h, 1.5 volumes of 0.8% lactic acid and without the second steeps. The best combination, a 95% decrease in InsP$_6$ and a myo-inositol value of 2.23 µmol per g dry weight, was achieved using 48 and 50°C for $T_1$ and $T_2$, respectively, and 1.5 volumes of 0.8% lactic acid. Under the latter conditions the final contents of InsP$_6$, InsP$_5$, InsP$_4$, InsP$_3$, InsP$_2$ and InsP were 0.66, 0.15, 0.79, 1.73, 1.1 and 0.9 µmol per g dry weight, respectively. Germination is responsible for the InsP$_6$ breakdown in malting processes for legumes and grains. Trugo et al. (1999) studied the effect of malting on the inositol phosphates in soybean, black bean, chickpea and barley seeds. After 2 days of malting the sum of InsP$_5$ and InsP$_6$ was reduced by 25% in black beans, which had the greatest losses. Higher levels of InsP$_3$ and InsP$_4$ were generated during malting, but InsP$_6$ was the predominant inositol phosphate present in all of the malted products. Additional reports on seed germination showed that little of the inositol phosphates in lentils was lost after 3 days but more than 70% was hydrolyzed by day 6 (Ayet et al., 1997), and 22–38% of the inositol phosphates in two species of lupin was destroyed in 4 days (de la Cuadra et al., 1994). Slight increases in InsP$_3$ and InsP$_4$ were observed during germination of the lentils and one of the species of lupin. Valencia et al. (1999) compared soaking and fermentation of raw and germinated quinoa flours followed by cooking. Cooking alone lowered the InsP$_6$ content of raw flour from 8.6–11.4 µmol/g to 6.9–9.5 µmol/g. Soaking in a suspension with three parts of water for 12–14 h at room temperature prior to cooking lowered the InsP$_6$ to 2.0–4.3 µmol/g, whereas fermentation with Lactobacillus plantarum prior to cooking gave 1.0–2.0 µmol/g InsP$_6$. The most extensive reduction of InsP$_6$ was in germinated flour that was fermented prior to cooking, which gave 0.2–0.3 µmol/g InsP$_6$, 0.0 µmol/g InsP$_5$ and 0.0–0.1 µmol/g each of InsP$_4$ and InsP$_3$. Soaking of maize flour or pounded maize was recently found to reduce the InsP$_5$ and InsP$_6$ content by more than half and was preferable to fermentation because the conditions were easier to control (Hotz and Gibson, 2001).

V. INOSITOL PHOSPHATES IN FRUITS AND VEGETABLES

Extremely little is known about the inositol phosphates in fruits and vegetables. Data obtained using nonspecific methods for phytate analysis
indicate that the amounts of InsP_6 in these foods are considerably lower than in seeds. A notable exception is avocado fruit, which contains approximately 0.5% InsP_6 on a dry weight basis (Phillippy and Wyatt, 2001). Avocado fruit, like seeds, contains a high level of fat that needs to be protected from oxidation. Therefore the fact that avocado fruit contains large amounts of InsP_6 bolsters the hypothesis that in vivo InsP_6 serves as an antioxidant to prevent iron-catalyzed free radical formation (Graf et al., 1987). Some vegetables that grow underground in the form of bulbs, roots and tubers may have higher amounts of inositol phosphates than green vegetables. Onions, parsnips and carrots have been reported to contain 0.38, 0.24 and 0.04% InsP_6, respectively, according to dry weight, whereas turnips, beet roots, celery and cabbage all had 0.02% or less InsP_6 (Harland and Morris, 1995). In all of these vegetables InsP_6 was the predominant inositol phosphate, and smaller amounts of InsP_5, InsP_4 and InsP_3 were also measured. Potatoes analyzed by NMR were reported to contain 0.09% InsP_6 (O’Neill et al., 1980), but HPLC results with several varieties of store-bought potatoes have revealed InsP_6 concentrations on average of about 0.3% on a dry weight basis (B. Q. Phillippy, unpublished data). Post-harvest changes in the inositol phosphates of these foods have never been studied, and it is not known whether the above data are typical for these foods or how much variation might be expected.

Plant cells also contain Ins(1,4,5)P_3, which is involved in calcium signaling and growth (Stevenson et al., 2000). Ins(1,4,5)P_3 is produced upon the hydrolysis of PtdIns(4,5)P_2 by phospholipase C following stimulation. Ins(1,4,5)P_3 then binds to receptors in the vacuole to release calcium stores, which evoke a biological response (Munnik et al., 1998). The levels of Ins(1,4,5)P_3 are low compared to those of InsP_6 and some of the other inositol phosphates. Red beets contain 8–11 pmol Ins(1,4,5)P_3/g of fresh weight (Beno-Moualem et al., 1995), and the stems of maize plants have 139–276 pmol Ins(1,4,5)P_3/g of fresh weight (Perera et al., 1999). In vegetative plant tissues Ins(1,4,5)P_3 appears to be a minor component of the InsP_3 fraction, which includes Ins(1,2,3)P_3, Ins(3,4,6)P_3, D- and/or L-Ins(1,5,6)P_3, D- and/or L-Ins(2,4,5)P_3 and D- and/or L-Ins(1,2,5)P_3 (Brearley and Hanke, 2000). Other inositol phosphates identified in Spirodela polyrhiza L. (duckweed) turions, which are similar to tubers, include Ins(3,4,5,6)P_4, Ins(1,3,4,5,6)P_5 and D- and/or L-Ins(1,2,4,5,6)P_5 (Brearley and Hanke, 1996).

VI. INOSITOL PHOSPHATES IN ANIMALS

A. ABSORPTION AND TISSUE CONTENT

The fate of inositol phosphates eaten by animals is particularly complex. Many factors interact as the inositol phosphates make their way through
the digestive tract to determine whether they will be excreted, degraded or absorbed. The composition of the diet may be most important, since food can contribute phytases and other phosphatases, minerals that affect the solubilities of the inositol phosphates, ionic components that can bind to the inositol phosphates, and components that may interact more indirectly. Nondietary factors that may also play a role in the destiny of the inositol phosphates include the genetic disposition of the animal as well as its physiological and nutritional status.

Significant enzymatic degradation of inositol phosphates can occur in the stomach of monogastric animals including humans if the right type and amount of phosphatases are present in the diet (Sandberg and Andersson, 1988; Sandberg et al., 1996; Mullaney et al., 2000). Additional phytases and phosphatases will be active in the intestines and may come from cells in the intestinal mucosa (Yang et al., 1991b; Maenz and Classen, 1998) or from microorganisms (Moore and Veum, 1983; Lopez et al., 1998). Adaptation to InsP$_6$ increased the phytase activity in the duodenum and ileum of rats, whereas wheat bran increased phytase activity only in the ileum (Lopez et al., 2000b). Resistant starch fed to rats adapted to wheat bran doubled the disappearance of InsP$_6$ in the feces, most likely by promoting the growth of digestive microflora that express phytase activity (Lopez et al., 2000a). Inositol liberated from inositol phosphates during digestion is actively absorbed in the small intestine and is transported through the blood for absorption by other tissues (Holub, 1982).

Inositol phosphates may have some limited ability to be absorbed, but the situation is far from clear. Sakamoto et al. (1993) found that [$^3$H]InsP$_6$ in drinking water appeared in gastric mucosal cells as a mixture of inositol and inositol mono- through hexakisphosphates, but only inositol and the monophosphate were detected in the blood plasma. Similarly, Vucenik and Shamsuddin (1994) showed that [$^3$H]InsP$_6$ incubated with HT-29 (human colon adenocarcinoma) cells appeared as a mixture of inositol and inositol mono- through hexakisphosphates in the cytosol. In contrast, YAC-1 (mouse lymphoma) and K562 (human erythroleukemia) cells exposed to [$^3$H]InsP$_6$ contained some of the less phosphorylated inositols but no detectable InsP$_6$. These results show that the cells contain phytase that can degrade InsP$_6$, but they do not show which compound(s) were absorbed, since the phytase and other phosphatases could have acted outside of the cells.

Grases et al. (2001a) recently demonstrated that the addition of dodecasodium phytate at a level of 1% by weight to diets devoid of InsP$_6$ and other forms of inositol for 12 weeks resulted in dramatic increases by more than ten-fold in the level of InsP$_6$ in the brain, liver, kidney, bone, urine and plasma of rats. Because inositol was not used as a control, it
could not be estimated how much of the tissue InsP$_6$ may have originated from synthesis via inositol liberated from InsP$_6$ by phytases in the gut. However, the results do show that tissue levels of InsP$_6$ respond to dietary manipulation in rats. The addition of InsP$_6$ to the rat diet resulted in a smaller increase in InsP$_5$ compared to InsP$_6$ in the tissues and fluids (Grases et al., 2001b), indicating that InsP$_6$ absorbed intact may have served as a precursor for the InsP$_5$.

In a study with human volunteers, Grases et al. (2001c) determined that the plasma levels of InsP$_6$ were normally 260±30 µg L$^{-1}$ but fell to 70±10 µg L$^{-1}$ after 15 days on a diet containing no cereal products, legumes, nuts, potatoes or coffee. Maximum concentration of InsP$_6$ in plasma was reached 4 h after ingestion of 1400 mg dodecasodium phytate, and urinary InsP$_6$ levels were directly related to plasma levels. It was noted that the overall percentage of absorption was low and that maximum urinary excretion, and thus maximum plasma levels, could be obtained by consumption of a diet containing normal amounts of InsP$_6$. Inositol levels in the diets were not measured nor was inositol used as a control to compare the effects of dietary inositol and dietary InsP$_6$ on InsP$_6$ levels in plasma and urine.

Adaptor protein 2 (AP-2) is an InsP$_6$ receptor associated with the plasma membrane (Voglmaier et al., 1992). By binding to AP-2, cytosolic InsP$_6$ prevents the binding of clathrin to AP-2 to form coated pits that take part in endocytosis (Gaidarov et al., 1996). However, the assembled coat structures containing clathrin and AP-2 have a greater affinity for di-octanoylphosphatidylinositol 3,4,5-trisphosphate than for InsP$_6$, suggesting that endogenous phosphoinositides occupy the AP-2 binding sites in the plasma membrane (Gaidarov et al., 1996). It is not known whether InsP$_6$ can be absorbed by endocytosis via these or other receptors.

Good evidence for the intact absorption of inositol phosphates was reported by Ozaki et al. (2000). Using polyamines including aminoglycosides such as neomycin, synthetic spherical dendrimeric polyamines with 12 or 32 primary amines and polybasic nuclear histone proteins as carriers, phosphoinositides or inositol phosphates were translocated into a variety of cells. Although the efficiency of cellular uptake was best for inositides with lipophilic moieties, an undetermined portion of 77 µM Ins(1,4,5)P$_3$ preincubated with 50 µM type III-S histone from calf thymus was taken up by NIH3T3 mouse fibroblasts and induced rapid cytosolic calcium mobilization. Thus polybasic compounds can neutralize the charge of inositol phosphates in a manner analogous to the intracellular delivery of oligonucleotides. Foods obtained from plants and animals contain a variety of compounds containing amines that could be tested for their ability to carry inositol phosphates into cells.
The large number of InsP₂, InsP₃ and InsP₄ isomers identified in animal cells has made quantification of their individual concentrations a tedious prospect. Some effort has been made to determine the amounts of those isomers which are most abundant. Ins(1,4,5)P₃ is present in animal cells at resting concentrations of about 0.1–2 µM with several-fold increases observed upon stimulation (Shears, 1989). Unstimulated levels of Ins(1,3,4)P₃ in some cells are 1–4 µM (Shears, 1989), whereas Ins(1,2,3)P₃ levels of 0.6–13.1 µM have been reported in various cell types (Barker et al., 1995).

The predominant inositol phosphates in most types of animal cells appear to be InsP₆ and Ins(1,3,4,5,6)P₅. In heart, kidney, spleen, liver and blood but not muscle of Buffalo rats InsP₆ and Ins(1,3,4,5,6)P₅ were typically present at levels of at least 5–15 nmol/g wet weight (Szwergold et al., 1987). InsP₆ levels alone were determined to be 1.04–2.80, 3.20–4.10, 30.0–42.0 and 1.07–1.21 µg/g in kidney, liver, brain and bone, respectively, of rats, and 0.14–0.31 and 0.43–2.52 µg/ml in plasma and urine, respectively, of humans (March et al., 2001). In various types of human blood cells InsP₆ ranged from 10 to 105 µM, whereas Ins(1,3,4,5,6)P₅ concentrations were 3–55 µM (Pittet et al., 1989; Bunce et al., 1993; Güse et al., 1993). Other isomers of InsP₅, InsP₄ and InsP₃ were present in these cells at lower levels, several of them between 1 and 10 µM. InsP₂ isomers were somewhat more abundant than InsP₄ or InsP₃, and the total monophosphates amounted to 27–93 µM (Bunce et al., 1993).

The inositol polyphosphate pyrophosphates consist of myo-inositol esters where five or six of the hydroxyl groups are substituted with different combinations of monophosphate and diphosphate groups yielding various configurations of InsP₆, InsP₇ and InsP₈. Radiolabeling experiments in AR4-2J pancreatoma cells indicates that their concentrations are probably low compared to concentrations of many of the other inositol polyphosphates (Shears et al., 1995).

Quick-frozen muscles from frogs and rats contained about 2–3 nmol InsP₆/g wet tissue (Table IV). Assuming that the muscles contained approximately 75% H₂O, the InsP₆ levels on a dry weight basis were about 10 nmol g⁻¹, which is 1000-fold less than the InsP₆ concentration found in raw seeds (Table II). After InsP₆, the most abundant inositol phosphate in muscle appears to be D- and/or L-Ins(1,4)P₂.

The inositol phosphate concentrations in animal products other than muscle used as food have been largely ignored owing to their low levels in comparison to the levels found in seeds. In fresh turkey blood the predominant inositol phosphate was Ins(1,3,4,5,6)P₅ at 1.1 mM, followed by 27 µM Ins(1,4,5,6)P₄, 6.6 µM InsP₆, 5.3 µM Ins(1,3,4,6)P₄, 1.8 µM Ins(1,5,6)P₃, 1.2 µM Ins(1,3,4,5)P₄, 1.1 µM Ins(2,4,5)P₃, 1.0 µM Ins(1,4,5)P₃ and 0.4 µM Ins(1,3,4)P₃ (Radenberg et al., 1989). In contrast, refrigerated
### TABLE IV
MASSES OF SOME INOSITOL PHOSPHATES IN SKELETAL MUSCLES

<table>
<thead>
<tr>
<th>Inositol Phosphate</th>
<th>Frog (nmol/g wet weight)</th>
<th>Rat (nmol/g wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>InsP&lt;sub&gt;6&lt;/sub&gt;</td>
<td>2.44–2.91</td>
<td>1.85–3.03</td>
</tr>
<tr>
<td>Ins(1,3,4,5,6)P&lt;sub&gt;5&lt;/sub&gt;</td>
<td>0.52–0.65</td>
<td>0.48–0.65</td>
</tr>
<tr>
<td>D- and/or L-Ins(1,2,4,5,6)P&lt;sub&gt;5&lt;/sub&gt;</td>
<td>0.14–0.24</td>
<td>&lt;0.02–0.15</td>
</tr>
<tr>
<td>D- and/or L-Ins(1,4,5,6)P&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.12–0.17</td>
<td>0.24–0.50</td>
</tr>
<tr>
<td>D- and/or L-Ins(1,2,5,6)P&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.03–0.05</td>
<td>&lt;0.03–0.06</td>
</tr>
<tr>
<td>D- and/or L-Ins(1,4,5)P&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1.21–1.46</td>
<td>0.69–1.48</td>
</tr>
<tr>
<td>D- and/or L-Ins(1,3,4)P&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.07–0.18</td>
<td>0.13</td>
</tr>
<tr>
<td>Ins(1,3,5)P&lt;sub&gt;3&lt;/sub&gt; and/or Ins(2,4,6)P&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.05–0.13</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>D- and/or L-Ins(1,4)P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>2.03–2.69</td>
<td>3.29–4.07</td>
</tr>
<tr>
<td>Ins(1,3)P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.28–0.87</td>
<td>1.33–1.85</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data compiled from Mayr and Thieleczek (1991).

calf brains contained mostly InsP<sub>6</sub> followed by Ins(1,3,4,5,6)P<sub>5</sub> (Phillippy and Bland, 1988). Interestingly, in rats injected with [<sup>3</sup>H]inositol, more labeled InsP<sub>6</sub> was present in the hippocampus after 24 h than in the other regions of the brain (Vallejo <em>et al.</em>, 1987). The intracellular level of InsP<sub>6</sub> in the rat hippocampus was estimated to be 13 µM, and similar concentrations were detected in the cerebellum, cortex and striatum (Yang <em>et al.</em>, 2001).

### B. BIOLOGICAL FUNCTIONS

All of our knowledge about the biological functions of inositol phosphates has come about within the last twenty years (reviewed in Irvine and Schell, 2001). In 1983 Streb and coworkers discovered that Ins(1,4,5)P<sub>3</sub>, which is formed by the action of phospholipase C on PtdIns(4,5)P<sub>2</sub>, releases calcium from a nonmitochondrial source in pancreatic acinar cells (Streb <em>et al.</em>, 1983). Subsequently other inositol phosphates were identified and their metabolic relationships were elucidated. In addition to calcium mobilization, a variety of other signaling functions have been associated with certain inositol phosphates (Shears, 1998). In particular, InsP<sub>6</sub> seems to be involved in numerous cellular processes as a result of its proclivity to bind to cationic minerals and proteins.

Binding of ligands such as hormones, neurotransmitters and growth factors to their receptors in the plasma membrane causes the hydrolysis of PtdIns(4,5)P<sub>2</sub> by phospholipase C to yield diacylglycerol and Ins(1,4,5)P<sub>3</sub>, which translocates through the cytoplasm as a second messenger (Berridge, 1993). Ins(1,4,5)P<sub>3</sub> receptors are calcium channels found in the
membranes of cellular organelles including the endoplasmic reticulum (Taylor et al., 1999), sarcoplasmic reticulum (Tasker et al., 2000), nuclear membrane (Humbert et al., 1996) and plasma membrane (Tanimura et al., 2000). Upon binding, Ins(1,4,5)P\textsubscript{3} activates its receptor, which results in the opening of the calcium channel and the release of stored calcium or uptake of extracellular calcium. Many cellular processes including growth, fertilization, secretion, contraction and sensation have been linked to Ins(1,4,5)P\textsubscript{3} signaling (Berridge, 1993). In addition, Ins(1,4,5)P\textsubscript{3} is involved in the regulation of cellular proliferation and apoptosis through this pathway (Patel et al., 1999; Jayaraman and Marks, 2000).

Different inositol phosphates can bind to and activate Ins(1,4,5)P\textsubscript{3} receptors in Xenopus oocytes or Chinese hamster ovary cells. Some metabolites of Ins(1,4,5)P\textsubscript{3} such as Ins(1,3,4,5)P\textsubscript{4} and Ins(1,3,4,6)P\textsubscript{4} display activity, although they are less potent than the former (Delisle et al., 1994; Burford et al., 1997). Highly active isomers that are not naturally abundant include Ins(4,5)P\textsubscript{2}, Ins(2,4,5)P\textsubscript{3}, DL-Ins(1,4,6)P\textsubscript{3} and DL-Ins(1,2,4,5)P\textsubscript{4}. Interestingly, some isomers formed by plant phytases, such as Ins(1,2,3)P\textsubscript{3}, may have a low calcium-releasing activity. Other isomers that released calcium from Xenopus oocytes included DL-Ins(1,2,6)P\textsubscript{3}, DL-Ins(1,5,6)P\textsubscript{3}, DL-Ins(1,2,3,6)P\textsubscript{4}, DL-Ins(1,2,5,6)P\textsubscript{4} and DL-Ins(1,2,3,5,6)P\textsubscript{5}, although the relative activities of the enantiomers in these pairs were not determined (Delisle et al., 1994).

In addition to membrane-bound receptors, inositol phosphates also interact with soluble proteins. Ins(1,4)P\textsubscript{2} binds to and activates 6-phosphofructokinase (Mayr, 1989). Ins(1,4)P\textsubscript{2}, Ins(1,4,5)P\textsubscript{3} and Ins(1,3,4,5)P\textsubscript{4} bind to and inhibit fructose 1,6-bisphosphate aldolase A (Koppitz et al., 1986). Aldolase-bound Ins(1,4,5)P\textsubscript{3} may also act as a pre-existing pool of the second messenger Ins(1,4,5)P\textsubscript{3} that is discharged by fructose 1,6-bisphosphate during glycolysis in muscle (Thieleczek et al., 1989).

Ins(1,2,6)P\textsubscript{3} is known to be produced from Ins(1,2,5,6)P\textsubscript{4} by phytases from plants and microbes (Phillippy, 1989; Türk et al., 2000). D- and/or L-Ins(1,2,6)P\textsubscript{3} has been identified as a minor inositol trisphosphate in some animal cells (McConnell et al., 1992). Also known as the pharmaceutical drug \(\alpha\)-trinositol, Ins(1,2,6)P\textsubscript{3} has analgesic and anti-inflammatory properties and is an antagonist of neuropeptide Y (Bell and McDermott, 1998). Although its mode of action is unknown, Ins(1,2,6)P\textsubscript{3} may inhibit signal transduction by binding to proteins such as receptors for Ins(1,3,4,5)P\textsubscript{4} (Bell and McDermott, 1998).

Ins(1,2,3)P\textsubscript{3} contains the functional 1,2,3-trisphosphate grouping that binds iron such that it cannot catalyze the formation of hydroxyl free radicals (Hawkins et al., 1993; Spiers et al., 1995, 1996). While acting as intracellular antioxidants, Ins(1,2,3)P\textsubscript{3} and other inositol phosphates containing this
grouping might safely transport iron between sites within the cell (Barker et al., 1995). Chelation of iron to the 1,2,3-trisphosphate grouping may also reduce the likelihood for lipid peroxidation catalyzed by iron bound to other anionic compounds (Phillippy and Graf, 1997). *In vitro* Ins(1,2,3)P$_3$ was nearly as effective as InsP$_6$ at preventing iron-catalyzed hydroxyl radical formation (Spiers et al., 1995, 1996), whereas InsP$_6$ was significantly better than Ins(1,2,3)P$_3$ at inhibiting iron-catalyzed lipid peroxidation (Phillippy and Graf, 1997).

Ins(1,3,4,5)P$_4$ appears to act synergistically with Ins(1,4,5)P$_3$ in the mobilization of calcium from intracellular stores. However, results from different studies have been variable and its mechanism is unclear (Smith et al., 2000). Low concentrations of Ins(1,3,4,5)P$_4$ may facilitate calcium influx by inhibiting Ins(1,4,5)P$_3$ 5-phosphatase, whereas higher concentrations may inhibit calcium signaling by binding to Ins(1,4,5)P$_3$ receptors (Hermosura et al., 2000). Ins(1,3,4,5)P$_4$ and other inositol phosphates with structural similarities to PtdIns(3,4,5)P$_3$ also compete with the latter for binding to proteins containing pleckstrin homology (PH) domains. This helps to regulate the recruitment of signaling molecules containing PH domains such as Gap1, protein kinase B (also known as Akt) and phospholipase C to cellular membranes (Kavran et al., 1998).

Ins(3,4,5,6)P$_4$ inhibits chloride secretion by epithelial cells following prolonged stimulation of Ins(1,4,5)P$_3$ production (Vajanaphanich et al., 1994). Receptor-mediated inositol phosphate turnover increases the conversion of Ins(1,3,4,5,6)P$_5$ to Ins(3,4,5,6)P$_4$, which inactivates chloride channels in the plasma membrane (Xie et al., 1996). In human pancreatic epithelial cells, Ins(3,4,5,6)P$_4$ specifically attenuates the longer term activation of calcium-dependent chloride channels by type II calmodulin-dependent protein kinase following the acute phase of calcium mobilization (Ho et al., 2001). Deficits in chloride channel activity regulated by Ins(3,4,5,6)P$_4$ may be involved in the kidney and lung pathology resulting from diabetes and cystic fibrosis, respectively (Ismailov et al., 1996). However, effective Ins(3,4,5,6)P$_4$ concentrations may be essential for the chloride ion regulation of metabolic functions such as the salt and fluid secretion of intestinal epithelial cells (Vajanaphanich et al., 1994).

Ins(1,3,4,5,6)P$_5$ has not been assigned any specific functions other than being a key intermediate in the formation of Ins(3,4,5,6)P$_4$ and InsP$_6$, and more recently as a substrate for the tumor suppressor protein PTEN (phosphatase and tensin homolog deleted on chromosome ten), which is a protein phosphatase, a PtdIns(3,4,5)P$_3$ 3-phosphatase and an Ins(1,3,4,5,6)P$_5$ 3-phosphatase (Caffrey et al., 2001). However, its structural similarity to certain InsP$_4$ isomers and InsP$_6$ results in some sharing of functionality. For example, both Ins(1,4,5,6)P$_4$ and Ins(1,3,4,5,6)P$_5$ interact strongly.
with the PH domain of protein kinase B (Razzini et al., 2000). Similarly, both Ins(1,3,4,5,6)P₅ and InsP₆ bind L- and P-selectins (Cecconi et al., 1994). In birds and some reptiles Ins(1,3,4,5,6)P₅ binds to hemoglobin and regulates its affinity for oxygen (Gersonde and Ganguly, 1986). InsP₆ has the ability to serve the same function, but neither inositol phosphate is naturally present in mammalian erythrocytes in sufficient amounts. Therefore, InsP₆ has been incorporated into human red blood cells in order to increase the delivery of oxygen to tissues (Boucher et al., 1996).

Numerous roles for InsP₆ in cells have been suggested to date (Shears, 2001), and it is very likely that more remain to be discovered. With its high negative charge density, much of InsP₆ is probably bound to cellular membranes through bridges of metal cations such as Mg²⁺ and Fe³⁺ (Poyner et al., 1993). By chelating iron in a manner that prevents it from catalyzing the formation of hydroxyl free radicals, InsP₆ may be a critical antioxidant (Graf and Eaton, 1990; Hawkins et al., 1993). Some of the proteins InsP₆ strongly binds to include L- and P-selectins (Cecconi et al., 1994), AP-2 (Timerman et al., 1992; Vogelmaier et al., 1992), AP-3 (Norris et al., 1995; Ye et al., 1995), coatomer (Fleisher et al., 1994), synaptotagmin (Fukuda et al., 1994; Linas et al., 1994), myelin proteolipid protein (Yamaguchi et al., 1996) and guanylate cyclase (Suzuki et al., 2001). A protein kinase stimulated by InsP₆ phosphorylates pacsin/syndapin I and thereby increases its association with dynamin I at nerve terminals (Hilton et al., 2001). InsP₆ also binds to PH domains, although with less affinity than some of the other inositol phosphates (Kavran et al., 1998). In the hippocampus, cerebellum, cortex and striatum regions of rat brain, InsP₆ levels were elevated upon activation and lowered by inhibition of neuronal activity (Yang et al., 2001).

Specific functions of InsP₆ in insulin-secreting cells are derived through the inhibition of phosphatases and the activation of protein kinase C. By inhibiting serine-threonine phosphatases, InsP₆ may enhance phosphorylation of voltage-gated L-type calcium channels resulting in calcium influx over the plasma membrane (Larsson et al., 1997). This in turn leads to an increase in cytoplasmic free calcium and insulin release. Activation of protein kinase C by InsP₆ may also lead to insulin secretion by promoting the recruitment and transport of granules to the site of exocytosis or by altering the conformation of proteins responsible for vesicle fusion (Efanov et al., 1997). Enhancement of calcium influx by InsP₆ has also been observed in other cells and organelles such as cerebellar neurons and liver mitochondria (Nicoletti et al., 1989; Copani et al., 1991). Recently, InsP₆ was observed to increase L-type calcium channel activity in hippocampal neurons by increasing the activity of adenyl cyclase, which raised cyclic AMP levels, which in turn enhanced the activity of protein
kinase A (PKA) (Yang et al., 2001). Thus L-type calcium channel activity in hippocampal neurons may be enhanced by InsP₆ through increased phosphorylation at PKA phosphorylation sites of the channel, in addition to the inhibition of serine/threonine protein phosphatases.

InsP₆ is also required for the export of mRNA from the nucleus to the cytoplasm, where it can be translated into protein (York et al., 1999; Feng et al., 2001). It is possible that InsP₆ functions by binding proteins associated with the nuclear pore complex or the shuttling heterogeneous nuclear ribonucleoprotein complexes. The three enzymes needed for synthesis of the InsP₆ required for mRNA export in Saccharomyces cerevisiae were phospholipase C, Ins(1,4,5)P₃ 6-kinase/Ins(1,4,5,6)P₄ 3-kinase and Ins(1,3,4,5,6)P₅ 2-kinase (York et al., 1999; Saiardi et al., 2000a). The intermediate Ins(1,4,5,6)P₄ was also shown in S. cerevisiae to be needed to regulate the transcription of six genes involved in the synthesis or breakdown of arginine (Odom et al., 2000).

The requirement of InsP₆ for efficient DNA repair of double-stranded breaks is an additional nuclear function that was recently discovered (Hanakahi et al., 2000). InsP₆ is part of the nonhomologous end-joining apparatus that consists of the XRCC4/DNA ligase IV complex and DNA-dependent protein kinase (DNA-PK), which is comprised of a catalytic subunit and the DNA end-binding protein Ku. InsP₆ binds to DNA-PK, but the mechanism by which InsP₆ promotes end-joining is unknown. Maximum activity was obtained with 1 µM InsP₆, although Ins(1,3,4,5,6)P₅ and Ins(1,3,4,5)P₄ were also somewhat effective ligands.

Ins(1,3,4,5,6)P₅ and InsP₆ can have phosphates enzymatically added onto one or two of their carbon-bound phosphates to form diphosphorylated inositol phosphates such as InsP₇ and InsP₈. These inositol pyrophosphates are found in animals, plants and microbes and thus far include [PP]₂-Insp₄, PP-InsP₅, [PP]₂-InsP₃ and PP-InsP₄ (Yang et al., 1999; Saiardi et al., 2000b). InsP₆, PP-InsP₅, and [PP]₂-InsP₄ are synthesized by Ins(1,3,4,5,6)P₅ 2-kinase, InsP₆ kinase and PP-InsP₅ kinase, respectively, which can also catalyze their reverse reactions to produce ATP (Phillippy et al., 1994; Voglmaier et al., 1996; Huang et al., 1998). Thus InsP₆ and the inositol pyrophosphates may function partly as cellular energy stores. InsP₇ and/or InsP₈ are involved in the homologous recombination mode of repair of double-stranded DNA breaks (Luo et al., 2002). The family of diphosphoinositol polyphosphates may regulate assembly of vesicles used for endocytosis and the trans-Golgi transport of proteins (Saiardi et al., 2000b), and InsP₇ may also have a role in vesicle exocytosis (Luo et al., 2001). In addition, the fact that InsP₆ kinase stimulates the uptake of inorganic phosphate may mean that InsP₇ or InsP₈ also help to regulate that process (Schell et al., 1999). An especially intriguing new finding is
that the post-transcriptional induction of an \(\text{InsP}_6\) kinase is largely responsible for the growth inhibition and apoptosis of human ovarian carcinoma cells following treatment with interferon-\(\beta\) (Morrison et al., 2001).

VII. NUTRITIONAL IMPORTANCE OF INOSITOL PHOSPHATES

A. BIOAVAILABILITY OF MINERALS

The history of the inositol phosphates from a nutritional perspective has followed an uneven path. As described in the review by Reddy et al. (1989), the propensity of \(\text{InsP}_6\) to impair the absorption of minerals such as calcium and iron was recognized early in the twentieth century. By chelating and precipitating multivalent cationic minerals, \(\text{InsP}_6\) was found to lower the bioavailability of the macrominerals calcium and magnesium as well as trace minerals such as iron and zinc. Since the precipitated mineral complexes contained \(\text{InsP}_6\), the inositol and phosphate moieties of this compound would also be unavailable for absorption, although this has not been viewed as a significant concern for humans. A change in the paradigm started in the 1980s when some positive nutritional attributes of \(\text{InsP}_6\) were discovered. Presently, nutritionists are attempting to clarify the roles of inositol phosphates in the diet and to determine the most appropriate levels for consumption.

There is general agreement that diets containing high levels of \(\text{InsP}_6\) can reduce the bioavailability of polyvalent cationic minerals (Zhou and Erdman, 1995; Rickard and Thompson, 1997; Harland and Narula, 1999). What is not clear is how much \(\text{InsP}_6\) is too much. As data on this subject have grown more extensive, it has become apparent that a simple rule cannot encompass all situations. Instead, special considerations must be made for different minerals, different groups of people and diets of different composition.

Although phytate can probably chelate all polyvalent cationic minerals, some minerals have received more attention than others in rough correlation to their perceived nutritional importance. Zinc and iron have been of primary concern because they readily form poorly available insoluble complexes with \(\text{InsP}_6\) and are critical for growth and development (Zhou and Erdman, 1995). Calcium has also received considerable attention, but it is now recognized that \(\text{InsP}_6\) inhibits calcium absorption less than oxalic acid does (Heaney et al., 1991; Frossard et al., 2000). Interactions of \(\text{InsP}_6\) with magnesium, copper, selenium, manganese, cobalt, nickel, cadmium, lead, aluminum and mercury have also been studied to varying degrees.
and cannot be ignored when considering the nutritional importance of inositol phosphates.

*In vivo* studies with Japanese quail, rats and humans have shown that moderate amounts of InsP$_6$ and InsP$_5$ decrease the absorption of zinc, whereas InsP$_4$ and InsP$_3$ have no effect (Tao *et al*., 1986; Lönnervald *et al*., 1989; Sandström and Sandberg, 1992). In Caco-2 cells, the inhibition of the uptake and transport of zinc was directly proportional to the number of phosphate groups on InsP$_3$, InsP$_4$, InsP$_5$ and InsP$_6$ (Han *et al*., 1994). *In vitro* studies showing that InsP$_6$ and InsP$_5$ bind more zinc than InsP$_4$ and InsP$_3$ at an inositol phosphate to zinc molar ratio of 1 : 14 indicated that chelation and precipitation are most likely the mechanisms responsible for inhibiting zinc absorption (Persson *et al*., 1998). Simpson and Wise (1990) also found that zinc was more soluble in the presence of InsP$_3$ and InsP$_4$ than InsP$_5$ and InsP$_6$ at molar ratios of 1 : 1, but at higher ratios of inositol phosphate to zinc the highest percentage of soluble zinc was observed in the presence of InsP$_6$. However, raising the molar ratio of calcium to zinc above 15 : 1 resulted in lower zinc solubilities in the presence of any of those inositol phosphates. In recent studies in rats and humans, respectively, whole wheat flour and oat bran, which have high levels of InsP$_6$, decreased the fractional absorption of zinc in comparison with low-fiber diets but enhanced the total zinc absorption owing to the high zinc contents of those ingredients (Levrat-Verny *et al*., 1999; Sandström *et al*., 2000). In rats fermentation of fibers in the large intestine increased the absorption of various minerals by increasing their solubility via a lower pH and microbial production of phytase (Lopez *et al*., 1998, 2000a). Thus the high zinc content of whole grains may compensate for the negative effect of InsP$_6$ on zinc absorption in some diets. Additional work is needed to determine whether these findings can be reproduced with other diets such as those containing legumes and the effects of different levels of calcium.

In a study of iron absorption from bread in humans, the sum of InsP$_3$, InsP$_4$, InsP$_5$ and InsP$_6$ was related to the inhibition of absorption (Brune *et al*., 1992). In experiments performed at molar ratios of inositol phosphate to iron of 0.04 : 1 to 3.6 : 1, the solubility of iron following *in vitro* digestion was decreased by InsP$_6$ and InsP$_5$ but slightly increased by InsP$_4$ and InsP$_3$ (Sandberg *et al*., 1989). The reason for the apparent different responses in the above studies to InsP$_4$ and InsP$_3$ in humans and *in vitro* was recently discovered. While InsP$_3$ and InsP$_4$ added individually to wheat rolls had no effect on iron absorption in humans, they did increase the negative effect of small amounts of InsP$_4$ and InsP$_6$ when all four were added together (Sandberg *et al*., 1999). Thus InsP$_3$ and InsP$_4$ potentiated the negative effect of InsP$_5$ and InsP$_6$, presumably by binding some of the soluble iron and
cross-linking it to insoluble complexes formed by iron and InsP_5 and InsP_6, thereby increasing the portion of iron that was insoluble. In Caco-2 cells 10:1 molar ratios of inositol phosphate to iron gave small differences in uptake of iron between InsP_3, InsP_4, InsP_5 and InsP_6, but the transport of iron across the cells was dramatically reduced in proportion to the number of phosphates (Han et al., 1994). Additional Caco-2 experiments with a 2:1 molar ratio of inositol phosphate to iron showed InsP_6 and InsP_5 inhibited iron uptake after 1 h, but various isomers of InsP_4 and InsP_3 only inhibited uptake after 4 h (Skoglund et al., 1999). Similar to zinc, whole wheat flour enhanced the total iron absorption in rats, but unlike with zinc, the per cent iron absorption was also increased relative to white wheat flour (Levrat-Verny et al., 1999). In an earlier rat study, Saha et al. (1994) found that the fractional absorption of iron and other minerals from whole wheat flour decreased with increasing phytate content, but was still high, 66% for iron, at the highest level of phytate. It would be helpful to do a similar study in humans. A consequence of the addition of dodecasodium phytate at a level of 1% to the diet of rats was a decrease in the iron concentration in their brains (Grases et al., 2001d). Degradation of InsP_6 by phytase has been used to increase the bioavailability of iron from soy-based infant formula and wheat bread (Davidsson et al., 1994; Sandberg et al., 1996).

Several strategies have been used to lower the amount of InsP_6 in seeds. Maize, barley, rice and soybeans with mutations in genes involved in InsP_6 synthesis have been developed with levels of InsP_6 reduced by about half or more (Larson and Raboy, 1999; Hatzack et al., 2000; Larson et al., 2000; Wilcox et al., 2000). When the low-phytic acid maize was used to make tortillas, iron absorption by men was 8.2% compared to 5.5% from tortillas made from wild-type maize (Mendoza et al., 1998). When porridge was prepared from the two types of maize, which had been fortified with additional iron, no effect on the iron absorption in women was observed (Mendoza et al., 2001). Because 92% of the activity of transgenic *Aspergillus fumigatus* phytase expressed in rice was lost after boiling the seeds for 20 min in water, phytase expression targeted to the endosperm to prevent the storage of phytic acid in the edible part of the seeds is currently under investigation (Lucca et al., 2001). Prospective work with an *E. coli* phytase introduced into *Arabidopsis* has produced transgenic seeds with reduced levels of phytate and increased free phosphate (Coello et al., 2001). Low-phytic acid grains and legumes have much anticipated potential for those who must satisfy their nutritional needs with a limited food intake and cannot supplement or fortify their diets with extrinsic minerals. A recent study unexpectedly found that variations in InsP_5 plus InsP_6 from 19.6 to 29.2 µmol/g in 24 genotypes of beans (*Phaseolus*
**vulgaris** L.) with 52–157 µmol/g of endogenous iron had no effect on iron bioavailability in rats (Welch *et al.*, 2000). However, the iron bioavailability from the beans was high, 53–76%, possibly due to unknown promoter substances and the rat intestinal phytase.

Two studies have compared the effects of various inositol phosphates on the availability of calcium. The absorption of calcium in fasted rats was 83% from a solution containing InsP₆, whereas InsP₅, InsP₄ and InsP₃ resulted in absorption of more than 98% of the dose at an inositol phosphate to calcium molar ratio of 4 : 1 (Lönnerdal *et al.*, 1989). In another rat study using purified inositol phosphate isomers, InsP₆ decreased calcium absorption, while Ins(1,2,3,4,5)P₅ and Ins(1,2,3,4,5,6)P₄ had no effect (Shen *et al.*, 1998). However, Ins(1,2,3,6)P₄ significantly increased calcium absorption at an inositol phosphate to calcium molar ratio of 1 : 25. It is possible that a portion of the Ins(1,2,3,6)P₄ entered cells and bound to receptors in the plasma membrane, thereby opening calcium channels, or this isomer may have formed a soluble calcium complex that was more readily absorbed. The bioavailability of calcium from grain and legume products such as whole wheat flour and soy flour is generally high (Mason *et al.*, 1993; Saha *et al.*, 1994), and components besides InsP₆ in wheat bran and beans may be more inhibitory to its absorption (Weaver *et al.*, 1993, 1996). In recent studies calcium transport across Caco-2 cell monolayers was reduced 16% by 2 mM InsP₆ (Kennefick and Cashman, 2000), and the fractional calcium absorption was decreased 16% by a diet containing 7.5 mmol/kg InsP₆ in rats (Harrington *et al.*, 2001).

Although the effects of different inositol phosphates on the bioavailability of polycationic minerals other than zinc, iron and calcium have not been investigated, it can be assumed that InsP₆ and InsP₅ will generally have the greatest negative impact. Interactions with other components of the diet, especially calcium and other minerals, also play an important role in determining availability. *In vivo* studies have continued to investigate concerns for the adverse effects of InsP₆ on the absorption of selenium and magnesium (Saha *et al.*, 1994; Pallauf *et al.*, 1998; Rimbach and Pallauf, 1999). In 10 mM metal chloride and 62.5 µM InsP₆ solutions at pH 6.0 and 7.0 more than half the Mg²⁺ precipitated within 2 h while all the Ca²⁺ remained in solution, but below pH 5.5 and 6.0, 10 mM Ca²⁺ and Mg²⁺, respectively, were 100% soluble at all concentrations of InsP₆ from 62.5 µM to 20 mM (Nolan *et al.*, 1987). In solutions of 1 mM InsP₆ and 1–3 mM copper(II) or zinc(II) ions at pH 5.9, copper(II) ions were more soluble than zinc(II) ions (Champagne and Hinojosa, 1987), which explains why InsP₆ can enhance the bioavailability of copper in the rat (Lee *et al.*, 1988). However, soluble zinc to copper molar ratios increased as the total metal ion to InsP₆ molar ratios increased from 2 : 1 to 12 : 1 (Champagne and Hinojosa, 1987), which
may help to explain why InsP$_6$ has also been found to inhibit copper absorption in rats (Lopez et al., 1998). In pH 7.0 solutions containing 10 mM InsP$_6$ and 1 mM metal ions, copper(II) remained soluble while zinc phytate slowly precipitated (Champagne and Fisher, 1990). Between pH 5 and pH 6, InsP$_6$, InsP$_5$, InsP$_4$ and InsP$_3$ can bind more copper than zinc, and the amount of metal bound is proportional to the number of phosphate groups (Persson et al., 1998). Additional mineral nutrients including manganese, cobalt and nickel are known to bind to InsP$_6$ (Vohra et al., 1965), and others such as chromium, molybdenum and vanadium are likely to do so as well.

Aluminum and the heavy metals also form complexes with inositol phosphates. Aluminum binds to the second messenger Ins(1,4,5)P$_3$ more strongly than to ATP, but the biological significance of this is not clear (Mernissi-Arifi et al., 1995). Aluminum and lead formed insoluble complexes with InsP$_6$ at metal to InsP$_6$ molar ratios of 5 : 1 to 3 : 1, whereas mercury was completely soluble at all InsP$_6$ concentrations (Bullock et al., 1995). At pH 5.0 in solutions containing 10 mM InsP$_6$ and 10 mM metal ions the solubilities of aluminum and lead were 69% and 4%, respectively. Mercury competes with calcium in binding to InsP$_6$ (Bullock et al., 1995) and forms complexes with Ins(1,2,6)P$_3$ (Lapp and Speiss, 1991). Thus one must consider the possibility that inositol phosphates might enhance the absorption of mercury, as has been shown for InsP$_6$ and copper. Cadmium binds to InsP$_6$, InsP$_5$, InsP$_4$ and InsP$_3$ less strongly than copper and zinc do at low pH values in the vicinity of pH 4 (Persson et al., 1998). In a recent study in rats, Aspergillus niger phytase addition to a maize-soybean diet to increase zinc absorption did not alter cadmium concentrations in the liver and kidney, but nonsignificant increases in femur lead were observed (Rimbach et al., 1998).

B. PREVENTION OF HEALTH DISORDERS

The backbone of most inositol phosphates in cells is myo-inositol. The nutritional importance of myo-inositol has long been recognized for its roles in the utilization of fat, as a growth promoter, and its ability to improve nerve conductance in diabetics (Holub, 1982, 1986). These functions may be partly or mostly derived from the use of inositol as a precursor of phosphatidylinositol and inositol phosphates. An extensive review of the metabolism of myo-inositol in plants was published recently (Loewus and Murthy, 2000). Inositol phosphates from seeds are a significant food source of myo-inositol, as are the phospholipids and free inositol from many plant- and animal-based foods (Berdanier, 1992). The
total myo-inositol contents of the majority of fruits, vegetables, grains and nuts analyzed after digestion with 6 N HCl for 40 h at 120°C were between 0.2 and 2.0 mg/g (Clements and Darnell, 1980). Myo-inositol has been evaluated for its ability to improve the mental health of patients with various psychiatric disorders (Kofman et al., 1998; Seedat and Stein, 1999; Kofman et al., 2000; Einat and Belmaker, 2001; Nemets et al., 2001). In addition to myo-inositol, smaller amounts of epi- and scyllo-inositol are present in human brains (Mclaurin et al., 2000). Phosphatidyl-scyllo-inositol appears to be synthesized more rapidly than phosphatidyl-mylo-inositol in barley seeds (Carstensen et al., 1999), but little is known about the metabolism or function of scyllo-inositol in animals. D-Chiro-inositol, which may be of benefit to diabetics (Steadman et al., 2000), and myo-inositol levels in urine of older men and women, appear to be related to insulin secretion (Campbell et al., 2001).

Myo-inositol and InsP₆ have synergistic or additive effects in inhibiting the development of cancer (Shamsuddin, 1999). In mice, dietary myo-inositol has been shown to be effective in preventing cancer of the colon (Shamsuddin et al., 1989), lung (Estensen and Wattenberg, 1993; Hecht et al., 1999; Wattenberg et al., 2000; Hecht et al., 2001), forestomach (Estensen and Wattenberg, 1993) and liver (Nishino et al., 1999). The anticancer action of InsP₆ is extensively documented as reviewed by Shamsuddin (1995, 1999) and Jenab and Thompson (2002). In rats, mice or humans InsP₆ has antitumor effects in cells or tissues of the blood, colon, liver, lung, mammary gland, prostate and skin. Although exogenous InsP₆ and wheat bran containing a similar amount of InsP₆ had similar effects on biomarkers of colon cancer risk in rats (Jenab and Thompson, 1998, 2000), the former was more effective in reducing the number of mammary tumors (Vucenik et al., 1997).

While most studies on the anticancer effects of InsP₆ have yielded positive results, a few contradictory reports have raised concerns regarding cancers of the urinary tract and rhabdomyosarcomas, which are muscle tumors occurring mainly in young people. Rats given drinking water containing 1.25% or 2.5% InsP₆ ad lib for 2 years passed blood in the urine from hemorrhage of necrotic renal papillae and developed renal papillomas (Hiasa et al., 1992). In rats given a combination of three cancer initiators and fed diets with or without 2% InsP₆ for 32 weeks, InsP₆ increased the incidence of urinary bladder papillomas (Hirose et al., 1999). However, in rats given a single different initiator and treated similarly with InsP₆ or its salts, InsP₆ alone had no effect while the dodecasodium salt of InsP₆ significantly increased the incidence of urinary bladder hyperplasias and papillomas (Hirose et al., 1999). It was concluded that the effect of InsP₆ itself was equivocal, but the dodecasodium salt of InsP₆ promoted carcino-
genesis. Alkaline salts are known to promote urinary bladder carcinogenesis by raising the urinary pH (Lina et al., 1994), which causes the formation of cytotoxic calcium phosphate precipitates (Cohen et al., 2000). In serum-free medium, micromolar levels of InsP\(_6\) stimulated the growth of two human rhabdomyosarcoma cell lines but inhibited the growth of a third rhabdomyosarcoma and two human colon carcinoma cell lines (Germain and Houghton, 1997). When rhabdomyosarcoma cells susceptible to growth inhibition by InsP\(_6\) were xenografted into nude mice, tumor size after 2 and 5 weeks was 25-fold and 49-fold smaller, respectively, in mice treated with InsP\(_6\) than in untreated controls (Vucenik et al., 1998). Additional studies to further clarify the potential risks associated with the consumption of InsP\(_6\) in regards to urinary tract cancers and rhabdomyosarcomas would be helpful.

There are a variety of mechanisms by which inositol and InsP\(_6\) may inhibit the development of cancer. There is evidence that myo-inositol suppresses the phosphatidylinositol 3-kinase pathway and thereby protects against the inhibition by carcinogens of cell differentiation (Jyonouchi et al., 1999). The effect of myo-inositol within the cell is likely to be mediated through one or more of its phosphorylated metabolites. Signal transduction through Ins(1,4,5)P\(_3\) is also elevated in human carcinomas, and inhibitors of phosphatidylinositol 4-kinase and phosphatidylinositol 4-phosphate 5-kinase induce differentiation and apoptosis of cancer cells (Weber et al., 1999). Razzini et al. (2000) propose that Ins(1,4,5,6)P\(_4\) and Ins(1,3,4,5,6)P\(_5\), but not InsP\(_6\), inhibit the growth of various types of cancer cells by binding to pleckstrin homology (PH) domains of Akt (protein kinase B), which is activated by the lipid products of phosphatidylinositol 3-kinase. It has been suggested that InsP\(_6\) inhibits cell transformation into a cancerous phenotype by directly inhibiting phosphatidylinositol 3-kinase (Huang et al., 1997; Dong et al., 1999) or by inhibiting the phosphorylation of extracellular signal-related protein kinases (Erks), c-Jun NH\(_2\)-terminal kinases (JNKs) or an inhibitor of nuclear factor κB (IκB) (Chen et al., 2001). Proteins that become upregulated following InsP\(_6\) treatment include p53 in HT-29 colon carcinoma cells (Saied and Shamsuddin, 1998) and hepatic glutathione S-transferase in mice (Singh et al., 1997). Ornithine decarboxylase, which is essential for the promotion of some tumors, is downregulated by InsP\(_6\) in mouse keratinocytes (Nickel and Belury, 1999). Furthermore, InsP\(_6\) inhibits endocytosis by impairing the binding of erbB1 to AP2 in prostate cancer cells (Zi et al., 2000). Although it seems possible that some InsP\(_6\) may enter cells intact, convincing proof for this is lacking. An alternative mechanism through which extracellular InsP\(_6\) conceivably could combat cancer is through mineral deprivation; intracellular zinc deficiency leads to cell death by apoptosis (Truong-Tran et al., 2000). Iron chelators such
as O-Trensox and desferrioxamine induce apoptosis in human hepatoblastoma HepG2 and hepatocarcinoma HBG cells, although addition of iron or zinc during treatment restores both proliferation and inhibition of apoptosis (Rakba et al., 2000). Another relevant observation is that InsP₆ enhances natural killer cell activity (Baten et al., 1989). InsP₆ is also a substrate for the InsP₆ kinase that transduces the signal from interferon-β for the growth inhibition and apoptosis of ovarian carcinoma cells (Morrison et al., 2001).

The antioxidant attributes of inositol phosphates may contribute to their anticancer activity as well as the prevention and amelioration of other conditions associated with excessive oxidation or inflammation. InsP₆ chelates iron within its 1,2,3-trisphosphate grouping, thus preventing iron-catalyzed hydroxyl free radical formation (Hawkins et al., 1993). Ins(1,2,3)P₃, D/L-Ins(1,2,3,4)P₄, Ins(1,2,3,5)P₄, b/h-Ins(1,2,3,4,5)P₅ and Ins(1,2,3,4,6)P₅ also possess this property (Hawkins et al., 1993; Spiers et al., 1995, 1996; Phillippy and Graf, 1997). InsP₃, InsP₄ and InsP₅ fractions derived from InsP₆ by hydrolysis with microbial phytase prevent the iron-catalyzed decomposition of lipid peroxides, which liberates peroxyl and/or alkoxyl radicals, whereas InsP₂ has no effect (Miyamoto et al., 2000). Studies have shown that dietary InsP₆ reduces lipid peroxide formation in the liver of lactating mice and the colon of pigs with high iron intake (Singh et al., 1997; Porres et al., 1999). In rats subjected to oxidative stress, dietary InsP₆ and Ins(1,2,3,6)P₄ decreased the production of lipid peroxides in the small intestine and colon, whereas only InsP₆ gave an antioxidative response in the lung (Burgess and Gao, 2002). However, InsP₆ had no effect on lipid peroxides, protein oxidation, α-tocopherol or reduced glutathione in the liver of growing rats (Rimbach and Pallauf, 1998). In HL-60 human leukemia cells and calf thymus DNA exposed to H₂O₂, InsP₆ reduced the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine, a biomarker of oxidative DNA damage; and cleavage at the 5′-guanine of GG and GGG sequences in c-Ha-ras-1 and p53 gene DNA fragments in the presence of H₂O₂ and copper was decreased by InsP₆ (Midorikawa et al., 2001). In vitro InsP₆ inhibited the oxidation of 6-hydroxydopamine by iron or manganese, accelerated catalysis by vanadium and had no effect in the presence of copper (Bandy et al., 2001). Formation of the p-quinone oxidation product proceeded most rapidly when the reduction potential of various metal-ligand complexes fell between the reduction potentials of 6-hydroxydopamine and molecular oxygen. Copper was the most effective catalyst, and the rate of 6-hydroxydopamine oxidation by copper phytate was increased three-fold in the presence of 100 mM Na₂SO₄.

The effectiveness of InsP₆ as an antioxidant food preservative has been demonstrated repeatedly (Empson et al., 1991; Lee and Hendricks, 1995;
Hix et al., 1997; Lee et al., 1998; Cornforth, 2002). Unlike iron, copper appears to bind preferentially to the 5-phosphate of InsP$_6$ (Champagne et al., 1990), but the ability of copper to produce hydroxyl free radicals is unhindered by InsP$_6$ (Madurawe et al., 1997). In contrast to InsP$_6$, the common food additive ethylenediaminetetraacetic acid (EDTA) stimulates iron-catalyzed but inhibits copper-catalyzed hydroxyl radical formation (Madurawe et al., 1997).

Ins(1,2,6)P$_3$, which is the drug α-trinositol and is produced by many plant and microbial phytases, exhibits analgesic and anti-inflammatory properties. It has been speculated that Ins(1,2,6)P$_3$ may function in part by binding to one or more of the proteins in the phosphatidylinositol signaling pathway (Bell and McDermott, 1998), but its effectiveness has only been reported for parenteral systemic administration and topical treatment of burned skin (Tarnow et al., 1998). It might be revealing to investigate the antioxidant and anti-inflammatory potencies of Ins(1,2,3)P$_3$ and Ins(1,2,6)P$_3$ in some model feeding studies.

InsP$_6$ lowers blood glucose, cholesterol and triglycerides (Rickard and Thompson, 1997; Jariwalla, 1999). This would be especially helpful for people susceptible to diabetes or heart disease. The mechanism for these effects is not completely clear but may be related to the inhibition of digestive enzymes by InsP$_6$ and/or a reduction in the plasma ratio of zinc to copper. Myo-inositol and InsP$_6$ were equally effective in preventing the elevation in liver lipids following sucrose feeding in rats (Katayama, 1999). It was proposed that both compounds worked by depressing hepatic lipogenesis rather than by inhibiting intestinal enzymes. Nonetheless, the old saying that a meal that keeps a person from getting hungry for a long time ‘sticks to the ribs’ may refer to foods high in InsP$_6$, which probably slows digestion by adhering to enzymes and other proteins in the lumen of the stomach and on the surface of the gastric mucosa. Indeed, α-amylase and lipase activities were significantly inhibited in vitro by 2–4 mM inositol phosphates containing one to six phosphates, and the decrease in digestibility and degree of inositol phosphorylation were highly correlated (Knuckles and Betschart, 1987; Knuckles, 1988).

Various other health benefits from InsP$_6$ consumption have been postulated. Some of those who eat diets high in red meat may accumulate excess iron, which can promote the development of infections, cancer and other degenerative diseases, and foods rich in InsP$_6$ may help prevent the over-accumulation of absorbed iron (Weinberg, 1999). This is a somewhat complex issue, especially in light of the recent observation that foods containing InsP$_6$ may lead to increased iron absorption simply because they increase the amount of iron consumed (Levrat-Verny et al., 1999). In some instances the iron from foods naturally high in InsP$_6$ may be less
available on a per cent basis than the iron in meat and thus less likely to be absorbed in overabundance. There has been an accumulation of evidence that dietary InsP₆ helps to prevent the formation of kidney stones (Zhou and Erdman, 1995). It has been suggested that InsP₆ excreted in the urine is responsible for preventing stone formation by inhibiting crystallization of calcium salts (Grases and Costa-Bauzá, 1999; Grases et al., 2000). However, 12 h following intragastric administration of [³H]InsP₆ to rats the radioactivity in the urine appeared to be associated with Ins and InsP but not with any of the inositol polyphosphates (Sakamoto et al., 1993). Thus any intact absorption and subsequent excretion of InsP₆ must be very low. InsP₆ has also been linked to upregulation of neutrophil functions (Eggleton, 1999) and the inhibition of platelet aggregation (Vucenik et al., 1999).

VIII. SUMMARY AND CONCLUSIONS

Advances in the analytical methods for inositol phosphates have enabled an increase in our knowledge of their nutritional roles in recent years. HPLC methods provide the separations needed to identify and quantify individual inositol phosphates in foods. The metabolic pathways for the synthesis and degradation of InsP₆ are multibranched and dependent upon the particular mix of enzymes and substrates present in the same cellular compartment. InsP₆ is the most abundant inositol phosphate in the raw seeds of most grains and legumes and generally is present at concentrations between 0.4 and 1.2% of the dry weight. InsP₆ and InsP₅ account for more than 95% of the total inositol phosphates in most raw grains and legumes and predominate in processed foods, which sometimes also contain substantial levels of InsP₄ and InsP₃. Avocado fruit and some vegetables contain appreciable amounts of InsP₆, but very little data is available in this area. Inositol phosphates appear to be mostly hydrolyzed to inositol prior to absorption in the guts of animals. Numerous inositol phosphate isomers in animal cells display an increasingly diverse range of biological functions. The fractional absorption of dietary minerals such as zinc and iron is decreased by InsP₆ and InsP₅, and these effects might be potentiated by InsP₄ and InsP₃. Myo-inositol and InsP₆ may help to prevent various health disorders by a number of possible mechanisms. More research is needed before the optimum levels of InsP₆ in the diets of people differing in age, sex and health concerns can be estimated.
IX. FUTURE RESEARCH NEEDS

The most immediate research need is to determine the appropriate levels of InsP$_6$ in human diets. Some InsP$_6$ may be desirable for its potential ability to prevent or delay various health disorders, but too much can result in mineral deficiencies. It may be helpful to establish a tolerance zone or range, which lies between the optimal and toxic doses, as has been suggested for essential trace elements such as selenium and chromium (Katz, 1996). This could be accomplished by feeding studies in which the effects of InsP$_6$ on indices of potential health benefits and on the bioavailabilities of minerals are monitored simultaneously. Since the tolerance zone likely depends on factors such as age, sex, health status and diet composition, experiments performed on different population groups would be necessary in order to be able to estimate the appropriate level of InsP$_6$ in someone’s diet. It will be important to identify trade-offs between the positive and negative nutritional aspects of InsP$_6$ if overlapping effects are observed and to attempt to resolve any potential conflicts. Then we will have a better idea of how much InsP$_6$ it would be prudent to remove from our foods by breeding or during processing.

More accurate data on the inositol phosphates in foods are needed. Since a number of inositol phosphates are bioactive and may be absorbed intact by cells under certain conditions, more data on their concentrations in foods should be obtained. The inositol phosphates and their natural variation in fruits and vegetables need to be analyzed, since comprehensive and accurate data for these foods is lacking. It might be a good idea to replace the nonspecific Association of Official Analytical Chemists’ method for phytate analysis with one of the HPLC methods if there is sufficient interest among researchers.

Biological studies are needed to clear up some lingering questions about the fate of inositol phosphates and their associated minerals within the gut. The possibility of adaptation to InsP$_6$ in the human diet needs to be investigated more thoroughly. Recent reports showing greater overall absorption of zinc and iron from diets containing high-phytate foods should be followed up with more definitive studies. The potential for absorption of inositol phosphates complexed with food components should be evaluated. It is not currently known to what extent any of the bioactive inositol phosphates consumed in foods can be absorbed before they are enzymatically degraded or whether there may be significant population group differences in inositol phosphate absorption.

Potential health benefits of myo-inositol and inositol phosphates in the diet have been identified. More research is needed to define their mechanisms of action and whether the effects are mediated predominately...
within the lumen of the gut or following absorption, on the surface of cells or intracellularly.

DISABLEMT

Mention of names of companies or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture over others not mentioned.

ACKNOWLEDGEMENT

The author thanks Dr John M. Bland for the computer-generated chemical structures.

REFERENCES


Crawton, A., Erneux, C. and Shears, S. B. 1994. Inositol 1,4,5,6-tetrakisphosphate is phosphorylated in rat liver by a 3-kinase that is distinct from inositol 1,4,5-trisphosphate 3-kinase. J. Biol. Chem. 269, 4337–4342.


Szwegold, B. S., Graham, R. A. and Brown, T. R. 1987. Observation of inositol pentakis- and


PYRROLIZIDINE ALKALOIDS IN FOODS

ROGER A. COULOMBE, JR

Graduate Program in Toxicology and
Department of Veterinary Sciences
Utah State University
Logan, UT 84322-4620
USA

I. Introduction
II. Plant Sources
III. Chemical Structures of Pyrrolizidine Alkaloids
IV. Pyrrolizidine Alkaloids in Foods and Herbal Medicines
   A. Pyrrolizidine Alkaloids in Foods
   B. Pyrrolizidine Alkaloids in Traditional Remedies and Medicines
V. Toxicity of Pyrrolizidine Alkaloids
VI. Metabolism of Pyrrolizidine Alkaloids
VII. Mechanism of Toxic Action
VIII. Control of Pyrrolizidine Alkaloids and Future Prospects
    Acknowledgements
    References

I. INTRODUCTION

In addition to the many well-known major nutrients (protein, fat, carbohydrate and fiber) and minor nutrients (vitamins, minerals and non-essential compounds), foods contain thousands of naturally present toxic plant compounds. Some are carcinogenic in animals, and thus may be potentially carcinogenic in people. Many of these compounds are commonly termed “nature’s pesticides” because they are often toxic to predators, such as insects and animals, thereby conferring a competitive advantage to the plant that produces them. Although these chemicals are in every meal we eat, they have received little attention compared to that given to the relatively minor residues of synthetic chemicals such as polychlorinated biphenyls (PCBs) and pesticides. Our food contains greater than 10 000-fold more natural toxins than the synthetic kind, and
in terms of metabolic reactions, our bodies are not able to distinguish between the two. Despite the popular notion equating “natural” and “healthy,” it is clear that natural toxins pose a far greater health risk than that posed by synthetic chemicals in our foods.

One important and well-known class of naturally occurring chemicals in foods is the pyrrolizidine alkaloids. Pyrrolizidine alkaloids are a large group of compounds; more than 350 pyrrolizidine alkaloids have been isolated from over 6000 plant species. The majority of pyrrolizidine alkaloids are toxic. Many of these have been shown to cause cancer in animals, and are therefore potentially carcinogenic in people.

People become intoxicated by pyrrolizidine alkaloids in a variety of ways. Epidemics of pyrrolizidine alkaloid poisoning have typically occurred when large numbers of people eat foods made from small grains contaminated with seeds from pyrrolizidine alkaloid-containing plants. Other less spectacular poisoning incidents occur from the use of certain dietary supplements and traditional “remedies,” direct consumption of pyrrolizidine alkaloid-containing plants, or through residues in food products, such as eggs, meat, milk and honey.

The real extent of human poisoning by pyrrolizidine alkaloids has probably gone woefully underreported. Pyrrolizidine alkaloid poisonings in people have been referred to as an “iceberg disease” because reported cases of pyrrolizidine alkaloid poisoning are probably only a small percentage of the true incidence (Huxtable et al., 1977). This is due to several factors, not the least of which is the difficulty inherent in determining the etiology of a chronic disease that often has a long latency period. In addition, the toxic effects have characteristics similar to those of other diseases, such as chronic alcoholism; pyrrolizidine alkaloids are seldom analyzed in dietary supplements and traditional remedies; and there has been a lack of medical inquiry stemming from the misconception that natural compounds are healthful rather than potentially harmful.

This is not intended to be an exhaustive review of all aspects of pyrrolizidine alkaloids. For that, I recommend Robin Mattock’s book Chemistry and Toxicology of Pyrrolizidine Alkaloids (Mattocks, 1986). Although it was published more than 15 years ago, it remains the definitive source for in-depth descriptions of nearly all aspects of pyrrolizidine alkaloids.

II. PLANT SOURCES

Pyrrolizidine alkaloids are produced by thousands of species of flowering plants in several higher plant families, but the genera responsible for most of the outbreaks of human poisonings are in Fabaceae (aka Leguminosae),
<table>
<thead>
<tr>
<th>Plant</th>
<th>Medicine (M) or food (F)</th>
<th>Country or region</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apocynaceae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asteraceae (Compositae)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Adenostyles alliariae</em></td>
<td>M</td>
<td>Italy</td>
<td>Sperl <em>et al.</em> (1995)</td>
</tr>
<tr>
<td><em>Ageratum conyzoides</em></td>
<td>M</td>
<td>China</td>
<td>Roeder (2000)</td>
</tr>
<tr>
<td><em>Cacalia decomposita</em></td>
<td>M</td>
<td>United States</td>
<td>Sullivan (1981)</td>
</tr>
<tr>
<td>(matarique)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cacalia hastate, hupensis</em></td>
<td>M</td>
<td>China</td>
<td>Roeder (2000)</td>
</tr>
<tr>
<td><em>C. yatabei</em></td>
<td>F</td>
<td>Japan</td>
<td>Hikichi <em>et al.</em> (1978)</td>
</tr>
<tr>
<td><em>Chromolaena odoranta</em></td>
<td>M</td>
<td>China</td>
<td>Roeder (2000)</td>
</tr>
<tr>
<td><em>Eupatorium cannabinum,</em></td>
<td>M</td>
<td>China</td>
<td>Roeder (2000)</td>
</tr>
<tr>
<td><em>chinense,</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>farfugium japonicum</em></td>
<td>M</td>
<td>Japan</td>
<td>Furuya <em>et al.</em> (1971)</td>
</tr>
<tr>
<td><em>Ligularia dentata</em></td>
<td>F</td>
<td>Japan</td>
<td>Asada and Furuya (1984)</td>
</tr>
<tr>
<td><em>Petasites japonicus</em></td>
<td>F, M</td>
<td>Japan</td>
<td>Hirono <em>et al.</em> (1973)</td>
</tr>
<tr>
<td><em>Senecio abyssinicus</em></td>
<td>M</td>
<td>Nigeria</td>
<td>Williams and Schoental (1970)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>M</td>
<td>United States</td>
<td>Wade (1977)</td>
</tr>
<tr>
<td><em>S. bupleuroides</em></td>
<td>M</td>
<td>Africa</td>
<td>Watt and Breyer-Brandwijk (1962)</td>
</tr>
<tr>
<td><em>S. burchelli</em></td>
<td>F, M</td>
<td>South Africa</td>
<td>Rose (1972)</td>
</tr>
<tr>
<td><em>S. coronatus</em></td>
<td>M</td>
<td>South Africa</td>
<td>Rose (1972)</td>
</tr>
<tr>
<td><em>S. discolor</em></td>
<td>M</td>
<td>Jamaica</td>
<td>Asprey and Thornton (1955)</td>
</tr>
<tr>
<td><em>S. doronicum</em></td>
<td>M</td>
<td>Germany</td>
<td>Roder <em>et al.</em> (1980)</td>
</tr>
<tr>
<td><em>S. inaequidens</em></td>
<td>F</td>
<td>South Africa</td>
<td>Rose (1972)</td>
</tr>
<tr>
<td><em>S. jacobaea</em></td>
<td>M</td>
<td>Europe</td>
<td>Schoental and Pullinger (1972); Wade (1977)</td>
</tr>
<tr>
<td>(ragwort)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. longilobus</em></td>
<td>M</td>
<td>United States</td>
<td>Stillman <em>et al.</em> (1977); Huxtable (1979)</td>
</tr>
<tr>
<td><em>S. monoensis</em></td>
<td>M</td>
<td>United States</td>
<td>Huxtable (1980)</td>
</tr>
<tr>
<td><em>S. nemorensis ssp. fuchsi</em></td>
<td>M</td>
<td>Germany</td>
<td>Habs <em>et al.</em> (1982)</td>
</tr>
<tr>
<td><em>S. pierottii</em></td>
<td>F</td>
<td>Japan</td>
<td>Asada and Furuya (1982)</td>
</tr>
<tr>
<td><em>S. retrorsus</em></td>
<td>M</td>
<td>South Africa</td>
<td>Rose (1972)</td>
</tr>
<tr>
<td>(S. latifolius)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. vulgaris</em></td>
<td>F</td>
<td>Japan</td>
<td>Hikichi and Furuya (1976)</td>
</tr>
<tr>
<td>(common groundsel)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Trichodesma africana</em></td>
<td>M</td>
<td>Asia</td>
<td>Omar <em>et al.</em> (1983)</td>
</tr>
<tr>
<td>Plant</td>
<td>Medicine (M) or Food (F)</td>
<td>Country or Region</td>
<td>Reference</td>
</tr>
<tr>
<td>-------</td>
<td>-------------------------</td>
<td>-------------------</td>
<td>-----------</td>
</tr>
<tr>
<td><em>Tussilago farfara</em> (coltsfoot)</td>
<td>M Japan; China?</td>
<td>Culvenor <em>et al.</em> (1976)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M Norway</td>
<td>Borka and Onshuus (1979)</td>
<td></td>
</tr>
<tr>
<td><strong>Boraginaceae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Anchusa officinalis</em></td>
<td>M Europe</td>
<td>Broch-due <em>et al.</em> (1980)</td>
<td></td>
</tr>
<tr>
<td><em>Arnebia euchroma</em></td>
<td>M China</td>
<td>Roeder (2000)</td>
<td></td>
</tr>
<tr>
<td><em>Cordia myxa</em></td>
<td>M China</td>
<td>Roeder (2000)</td>
<td></td>
</tr>
<tr>
<td><em>Cynoglossum amabile, lanceolatum, zeylanicum</em></td>
<td>M China</td>
<td>Roeder (2000)</td>
<td></td>
</tr>
<tr>
<td><em>C. geometricum</em></td>
<td>M East Africa</td>
<td>Schoental and Coady (1968)</td>
<td></td>
</tr>
<tr>
<td><em>C. officinale</em></td>
<td>M Iran</td>
<td>Coady (1973)</td>
<td></td>
</tr>
<tr>
<td><em>Hackelia floribunda</em> (western false forget-me-not)</td>
<td>M United States</td>
<td>Hagglund <em>et al.</em> (1985)</td>
<td></td>
</tr>
<tr>
<td><em>Heliotropium eichwaldii</em></td>
<td>M India</td>
<td>Datta <em>et al.</em> (1978a, b); Gandhi <em>et al.</em> (1966)</td>
<td></td>
</tr>
<tr>
<td><em>H. europaeum</em></td>
<td>M India; Greece</td>
<td>IARC (1976)</td>
<td></td>
</tr>
<tr>
<td><em>H. indicum</em></td>
<td>M India, Africa, South American, China</td>
<td>Schoental and Coady (1968); Hoque <em>et al.</em> (1976)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Roeder (2000)</td>
<td></td>
</tr>
<tr>
<td><em>H. ramossissimum</em> (ramram)</td>
<td>M Arabia</td>
<td>Macksad <em>et al.</em> (1970); Coady (1973)</td>
<td></td>
</tr>
<tr>
<td><em>H. supinum</em></td>
<td>M Tanzania</td>
<td>Schoental and Coady (1968)</td>
<td></td>
</tr>
<tr>
<td><em>Symphytum officinale</em> (comfrey)</td>
<td>F, M Japan (and elsewhere)</td>
<td>Hirono <em>et al.</em> (1978)</td>
<td></td>
</tr>
<tr>
<td><em>S. x uplandicum</em></td>
<td>F, M General</td>
<td>Hills (1976)</td>
<td></td>
</tr>
<tr>
<td><em>S. x uplandicum</em></td>
<td>M Europe</td>
<td>Roeder <em>et al.</em> (1992)</td>
<td></td>
</tr>
<tr>
<td><strong>Fabaceae (Leguminosae)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cassia auriculata</em></td>
<td>M, F Sri Lanka; India</td>
<td>Arseculeratne <em>et al.</em> (1981)</td>
<td></td>
</tr>
<tr>
<td><em>Crotalaria albina, assamica, mucronata,.sessiliflora, tetragona</em></td>
<td>M China</td>
<td>Roeder (2000)</td>
<td></td>
</tr>
<tr>
<td><em>C. brevidens</em></td>
<td>F East Africa</td>
<td>Coady (1973)</td>
<td></td>
</tr>
<tr>
<td><em>C. fulva</em></td>
<td>M Jamaica</td>
<td>Barnes <em>et al.</em> (1964); McLean (1970, 1974)</td>
<td></td>
</tr>
<tr>
<td><em>C. incana</em></td>
<td>M East Africa</td>
<td>Schoental and Coady (1968)</td>
<td></td>
</tr>
<tr>
<td><em>C. juncea</em></td>
<td>M, F India</td>
<td>Chopra (1933); Watt and Breyer-Brandwijk (1962)</td>
<td></td>
</tr>
<tr>
<td><em>C. laburnifolia</em></td>
<td>M Tanzania</td>
<td>Schoental (1968); Coady (1973)</td>
<td></td>
</tr>
<tr>
<td><em>C. mucronata</em></td>
<td>F Asia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M Tanzania</td>
<td>Coady (1973)</td>
<td></td>
</tr>
</tbody>
</table>
TABLE I (continued)
SOME PLANTS CONTAINING (OR SUSPECTED OF CONTAINING) PAs WHICH HAVE BEEN USED AS EITHER
HERBAL MEDICINES OR FOODS

<table>
<thead>
<tr>
<th>Plant</th>
<th>Medicine (M) or food (F)</th>
<th>Country or region</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. recta</em></td>
<td>M, F</td>
<td>Tanzania</td>
<td>Schoental and Coady (1968); Coady (1973)</td>
</tr>
<tr>
<td><em>C. retusa</em></td>
<td>M, F</td>
<td>Africa; India</td>
<td>IARC (1976); Watt and Breyer-Brandwijk (1962)</td>
</tr>
</tbody>
</table>

**Orchidaceae**
* Liparis nervosa
  M                     China     | Roeder (2000)

**Scrophulariaceae**
* Castilleja integra
  M                     Southwestern US | Mead *et al.* (1992)

* C. rhexifolia
  M                     Southwestern US | Stermitz and Suess (1978)

* Pedicularis sp.
  M                     Southwestern US | Schneider and Stermitz (1990)

Updated from Mattocks (1986).

Asteraceae (Compositae) and Boraginaceae (Table I). Pyrrolizidine alkaloid-producing plants are common worldwide, and are considered noxious weeds in many geographical areas. So abundant are pyrrolizidine alkaloids that they are estimated to be in 3% of the world’s flowering plants (Winter and Segall, 1989). While alkaloids are found in many parts of the plant, they are typically associated with seeds or fruit, but other parts may also contain pyrrolizidine alkaloids. In fact, the highest recorded pyrrolizidine alkaloid content of any plant, 18% dry weight, was found in the leaves of *Senecio riddellii* (Molyneux and Johnson, 1984).

**III. CHEMICAL STRUCTURES OF PYRROLIZIDINE ALKALOIDS**

The majority of pyrrolizidine alkaloids, and all of the ones discussed in this review, contain an eight-membered necine base, which can either be saturated or contain a double bond in the 1,2-position (Figure 1). The presence of this double bond is an important determinant in the hepatotoxicity of pyrrolizidine alkaloids in that only those with 1,2-unsaturation are hepatotoxic. Other variants of the necine base are also found, such as the

SOFTbank E-Book Center Tehran, Phone: 66403879,66493070 For Educational Use. www.ebookcenter.ir
discontinuous ring structure, otonecine, in petasitenine and senkirkine (Figure 2). The necine moiety is often esterified to constituents called necic acids, which vary significantly in structure. Necic acids may be absent (as in retronecine; Figure 2) or they may be present as open esters (heliosupine) or as macrocyclic esters (senecionine, monocrotaline, petasitenine and senkirkine). Other features of necic acids include an epoxide (petasitenine), saturation (monocrotaline) or unsaturated carbon nuclei (senecionine). Pyrrolizidine alkaloids with a cyclic diester moiety are generally more potently toxic, and are more likely to form more cellular DNA cross-links than the other forms (Kim et al., 1993, 1995). Usually coexisting in the plant with pyrrolizidine alkaloids, often in greater quantities, are N-oxides, such as indicine N-oxide (Figure 2), which often may represent the majority of the total pyrrolizidine alkaloid content. N-oxides usually become reduced to the basic alkaloids during the process of extraction.

IV. PYRROLIZIDINE ALKALOIDS IN FOODS AND HERBAL MEDICINES

Since pyrrolizidine alkaloid-containing plants are distributed worldwide, poisoning incidences are, predictably, seen in many geographical locations.
FIG. 2. Chemical structures of several representative pyrrolizidine alkaloids. Some pyrrolizidine alkaloids have necic acids esterified to the necine base. Necic acids may be absent (retronecine), or may be present as open esters (heliosupine) or as macrocyclic esters (senecionine, monocrataline, petasitenine and senkirkine). Other possible features of necic acids may include an epoxide (petasitenine), saturation (monocrataline) or unsaturated carbon nuclei (senecionine). N-Oxides, represented here by indicine N-oxide, usually coexist with the reduced pyrrolizidine alkaloid in the plant.
The majority of reports of outbreaks of pyrrolizidine alkaloid poisoning have largely been limited to third world countries. Generally, these have been outbreaks where hundreds, and sometimes thousands, have been poisoned from eating staple foods made from cereal crops contaminated with seeds from pyrrolizidine alkaloid-containing weeds. More recently, however, along with an increasing reliance on unconventional medicine and the use of herbal supplements and traditional medicines, there has been a sharp rise in the number of poisonings in industrialized countries. A survey of some of the plants reported to be responsible for incidences of human poisonings, whether from foods or medicines, is presented in Table II.

A. PYRROLIZIDINE ALKALOIDS IN FOODS

Numerous sporadic outbreaks of pyrrolizidine alkaloid intoxications have been recorded (Table II). These are largely attributed to consumption of staple small grains contaminated with seeds from Senecio, Heliotropium,

<table>
<thead>
<tr>
<th>Plant</th>
<th>PA (if identified)</th>
<th>Country or region</th>
<th>Number of cases</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Senecio ilicifolius; S. burchelli Senecio spp.</td>
<td>Senecionine? –</td>
<td>South Africa</td>
<td>80</td>
<td>Willmont and Robertson (1920) Selzer and Parker (1951)</td>
</tr>
<tr>
<td>Heliotropium lasiocarpum Crotalaria fulva</td>
<td>Heliotrine; lasiocarpine Fulvine</td>
<td>Central Asia; West Indies</td>
<td>28</td>
<td>McLean (1970)</td>
</tr>
<tr>
<td>Heliotropium lasiocarpum</td>
<td>Heliotrine</td>
<td>Central Asia</td>
<td>61</td>
<td>Bras et al. (1954); Bras and Hill (1956) McLean (1970)</td>
</tr>
<tr>
<td>Heliotropium popovii</td>
<td>Heliotrine</td>
<td>Afghanistan</td>
<td>7800</td>
<td>Mohabbat et al. (1976); Tandon et al. (1978)</td>
</tr>
<tr>
<td>Crotalaria nana</td>
<td>Crotananine; cronaburmine</td>
<td>India</td>
<td>67</td>
<td>Tandon et al. (1976), Siddiqi et al. (1978) Chauvin et al. (1993); Mayer and Luthy (1993)</td>
</tr>
<tr>
<td>Heliotropium popovii</td>
<td>Heliotrine</td>
<td>Tajikistan</td>
<td>3906</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Mattocks (1986).
Crotalaria and other genera of pyrrolizidine alkaloid-containing plants. As with many episodes of food intoxication caused by naturally occurring toxins from plants and fungi, they are often associated with consumption of a contaminated staple food supply that is locally grown.

Pyrrolizidine alkaloids are one of the few classes of natural toxins to be regulated in foods or herbal supplements. In 1992, the German Federal Health Agency established a maximum allowable daily intake of 0.1 µg of pyrrolizidine alkaloids and N-oxides in herbal supplements. One µg per day is allowed if the intake is limited to 6 weeks per year (German Federal Health Bureau, 1992; Edgar and Smith, 2000). The United States has no such regulation. Currently, there is no limit on intake of pyrrolizidine alkaloid-containing foods, herbal supplements or other products, or the amount of pyrrolizidine alkaloids that a herbal supplement can contain. Pyrrolizidine alkaloids enter the food chain in a variety of ways – through grain, milk, honey, eggs and herbal medicines. Because these items can at times be contaminated at concentrations that exceed the German regulations, a real risk is posed to human health by pyrrolizidine alkaloids in foods.

1. Pyrrolizidine alkaloids in staple foods

The first report of human poisonings due to pyrrolizidine alkaloids recorded in scientific literature occurred in 1918 in the George District of Cape Province, South Africa (Willmont and Robertson, 1920). Dubbed “Senecio disease,” it was apparently caused by contamination of cereal grains with seeds of Senecio ilicifolius and S. burchelli, which were made into bread. The problem appeared to be exacerbated by “old-fashioned” mills that did not efficiently winnow the grain, and the fact that bread was the staple diet of the largely poor populace. The outbreak was responsible for some 80 cases over a 10-year period, most of whom were children. The symptoms, which were often fatal, included nausea, vomiting, acute gastric pain, ascites and hepatic distension, and had an onset of 2 weeks to 2 years or longer. The investigators noted similarities in symptoms and post-mortem signs between this outbreak and Moltendo disease in South Africa, Winton’s disease in New Zealand, and Picton disease in Nova Scotia, all diseases in livestock known to be caused by Senecio.

Seltzer and Parker (1951) published details of clinical and post-mortem findings of Senecio poisoning in Cape Town, South Africa, similarly attributed to contamination of wheat with seeds from Senecio sp. Of the 12 cases described, six were fatal. The most common and most constant early symptoms in all patients were abdominal pain and swelling, followed by rapidly developing ascites and hepatomegaly. Surviving victims recalled
that the tainted bread had an abnormal taste, some describing it as “musty,” others as “bitter.” These investigators noted the similarity of these signs to those of Budd–Chiari syndrome, a hepatic disease characterized by obstruction of the trunk and large branches of the hepatic vein.

An outbreak of veno-occlusive disease (VOD) that was probably caused by consumption of cereals contaminated with seeds of *Crotalaria* sp. containing pyrrolizidine alkaloids occurred in the Sarguja district of India in November–December 1975 (Tandon *et al*., 1976). Forty-two per cent of the 67 recorded cases died. A follow-up study reported post-mortem signs characteristic of pyrrolizidine alkaloid poisoning, which included changes ranging from acute hemorrhagic centrilobular necrosis, progressive sclerosis to nonportal cirrhosis, and in the terminal phases, occlusion of the terminal veins (Tandon *et al*., 1977).

A much larger outbreak of VOD occurred in northwestern Afghanistan. This was caused by consumption of bread made from wheat contaminated with seeds of *Heliotropium*, which contained heliotrine (Figure 3) (Mohabbat *et al*., 1976; Tandon *et al*., 1978). Approximately 7800 were affected. In a small number of cases, clinical improvement was observed after several months of hospitalization.

Another extensive outbreak of pyrrolizidine alkaloid poisoning occurred in 1992 in southern Tajikistan, caused by consumption of bread contaminated with *Heliotropium popovi* (Chauvin *et al*., 1993; Mayer and Luthy, 1993). The outbreak was exacerbated by political instability that led to a blockade of the Farkhar region, causing a 2-month delay of the wheat harvest and subsequent famine. Because of this delay, *Heliotropium* had time to go to seed, and then contaminate the wheat at harvest. In the area affected by the outbreak, a poisoning rate of 4% occurred, and a total of 3906 cases of VOD was recorded. The overall fatality rate was approximately 1.3%. Attack rates favored younger children and adolescents, while the case–fatality rate increased with age. The most dominant alkaloid in samples of contaminated wheat was heliotrine (Mayer and Luthy, 1993).

2. Pyrrolizidine alkaloids in milk

Another potential source of pyrrolizidine alkaloids in the human food chain is from the milk of animals that have ingested pyrrolizidine alkaloid-producing plants. The pyrrolizidine alkaloid, jacoline, was detected in the milk of four lactating cows given *Senecio jacobaea* (tansy ragwort) via rumen canula at the dose rate of 10 g kg$^{-1}$ day$^{-1}$ for 2 weeks. The plants, which contained five pyrrolizidine alkaloids, had an average pyrrolizidine alkaloid content of 0.16% dry weight. The dosed cows exhibited marked changes in blood leukocyte count, sorbitol dehydrogenase values, and mild
FIG. 3. Selected pyrrolizidine alkaloids found in folk medicines and foods that have been involved in human poisoning incidents. Fulvine, from *Crotalaria fulva*, an ingredient in some “bush teas,” is responsible for endemic veno-occlusive disease (VOD) in the West Indies. Heliotrine, from *Heliotropium popvoii* and spp., is the etiological agent of large endemics of VOD in Afghanistan and Tajikistan. Seneciphylline, a potent hepatotoxin in *Senecio*-based teas, is extensively used in southwestern United States and Northern Mexico, where it is known under various names. It has also been found in honey. Intermedine is one of the major hepatotoxic alkaloids found in the widely used traditional herb comfrey *Symphytum officinale* and Siberian comfrey *S. uplandicum*. Lycopsamine is another major alkaloid in comfrey. Echimidine was found in commercial Australian honey made from bees pollinating *Echium plantagineum*. Australian researchers recently found lasiocarpine, among other alkaloids, in commercial eggs at concentrations ranging from 1.2 to 9.7 µg of total alkaloid per egg. Chickens were given a wheat-based feed grain contaminated with *Heliotropium europaeum*. Intermedine, lycopsamine and senecionine were found in *Liatris punctata*, also known as “cachana,” “grey feather” and “blazing star.” This herb is traditionally used by some Native Americans and Hispanics in the southwestern United States as a diuretic, for throat inflammation, laryngitis, cough and nosebleeds.
liver pathology, consistent with hepatotoxicity. However, no such changes were noted in calves nursing from these cows. Jacoline was found in concentrations ranging from 9.4 to 16.7 µg per 100 mL of milk (Dickinson et al., 1976). Milk from goats fed tansy ragwort (1% body weight per day) caused either a negative or a mildly positive mutagenic response in the Salmonella/mammalian microsome (Ames) assay (White et al., 1984). Another study confirmed that transfer of pyrrolizidine alkaloids in milk can cause hepatic toxicity. Rats fed a diet of milk from goats fed tansy ragwort (7.5 ng of pyrrolizidine alkaloid per g dry weight) for 180 days with a calculated total pyrrolizidine alkaloid intake of 0.96 mg per rat had swollen hepatocytes of centrilobular distribution and biliary hyperplasia, indicating hepatic toxicity due to pyrrolizidine alkaloids (Goeger et al., 1982). Importantly, these hepatic signs were similar to those found in rats fed tansy directly at concentrations of 1%, 0.1%, 0.01% and 0.001% (corresponding to pyrrolizidine alkaloid intakes of 39.77, 5.04, 0.52, and 0.05 mg per rat). In this study, no changes were noted in two calves fed the pyrrolizidine alkaloid-containing goats’ milk.

3. Pyrrolizidine alkaloids in honey

Honey is another food source found to contain pyrrolizidine alkaloids. Initial investigations found pyrrolizidine alkaloids at relatively low concentrations of 0.3–3.9 ppm in honey produced from bees foraging fields of S. jacobea (Deinzer et al., 1977). The type of alkaloids found in the honey – senecionine, seneciphylline, jacoline, jaconine, jacobine and jacozone – reflected those found in the local plant. In one study, Senecio pollen counts in honey samples were correlated with pyrrolizidine alkaloid concentrations. Honey that contained S. jacobea pollen (15–21 grains g⁻¹) with total alkaloid concentrations of 0.011–0.056 mg kg⁻¹ contained jacobine, jacozone, seneciophylline and senecionine. Honey samples containing two grains or less did not contain detectable alkaloids (Crews et al., 1997). These authors did not detect pyrrolizidine alkaloids in honey purchased in retail outlets.

Australian researchers discovered that honey produced from stands of the purple flower Echium plantagineum, known as “Patterson’s Curse” or “Salvation Jane,” contained a pyrrolizidine alkaloid content ranging between 0.27 and 0.95 ppm (Culvenor et al., 1981). The main alkaloid found in honey obtained from four suppliers in Victoria and New South Wales contained echimidine, with smaller amounts of 7-acetylycopsamine, 7-acetylintermedine, echimine, uplandicine, lycopsamine, intermedine (Figure 3) and a novel alkaloid. Given usual patterns of honey consumption and the relatively low concentrations of alkaloids found, there is probably little, if any, human health risk posed by pyrrolizidine alkaloids in honey.
4. Pyrrolizidine alkaloids in eggs

While there have been many instances of poultry being poisoned by pyrrolizidine alkaloids in their feed, one report has indicated that pyrrolizidine alkaloids can enter the human food chain via eggs (Edgar and Smith, 2000). Three flocks of chickens from a small-scale egg producer were given wheat-based feed grain contaminated with *Heliotropium europaeum*, at a concentration estimated to be 0.6% by weight. Analysis of the wheat uncovered the presence of the pyrrolizidine alkaloids supinine, heleurine, heliotrine, europine and lasiocarpine. These pyrrolizidine alkaloids (as well as others, probably as a result of metabolism) were found at concentrations ranging from 1.2 to 9.7 µg per egg.

5. Pyrrolizidine alkaloids in meat

Residues of pyrrolizidine alkaloids have not been found in meat from animals ingesting alkaloid-containing plants. There are several possible reasons for this. These compounds are metabolized into reactive pyrrolic intermediates with relatively short half-lives, which rapidly bind to cellular macromolecules, such as GSH, proteins and DNA. Thus, it is unlikely that sufficiently large amounts of residues of pyrrolizidine alkaloids *per se* would exist in meat products to be of any risk to the consumer. One possible exception to this would be if an animal is slaughtered only hours after grazing on contaminated pasture. In any event, it is not likely that pyrrolizidine alkaloids in meat pose a significant health threat.

B. PYRROLIZIDINE ALKALOIDS IN TRADITIONAL REMEDIES AND MEDICINES

In North America, where crop weeds are controlled by herbicides and foods regularly inspected, the biggest risk of exposure to pyrrolizidine alkaloids comes primarily from so-called herbal “remedies,” herbal teas and folk medicines (Huxtable, 1989). In contrast to the sporadic outbreaks of poisonings in many third world countries, poisonings from consumption of pyrrolizidine alkaloid-contaminated botanical folk medicines account for a consistent, regular occurrence of cases.

Taken as a whole, the majority of herbal drinks are harmless and in some cases may even be beneficial. However, some herbal teas that are widely sold in health food stores contain pyrrolizidine alkaloids that have been implicated in many cases of human poisonings.

The serious health risk posed by pyrrolizidine alkaloid-containing plant-based supplements compelled the German Federal Health Bureau to
establish regulations limiting intake. Unfortunately, in the United States, recent legislation has moved in the opposite direction, away from consumer protection. The ironically misnamed “Dietary Supplement Health and Education Act” of 1994, now exempts “natural” food products such as folk medicines and teas from safety regulations that normally apply to prescription drugs. In fact, this law placed dietary supplements into a new category, distinct from food or drugs, that is now exempt from the rules the US Food and Drug Administration used against questionable products. Protected by this legislation, manufacturers and dispensers of herbal supplements are not required to provide information on content, effectiveness (or lack thereof), or possible adverse effects. Furthermore, in the case of botanical supplements, there is usually no reliable information available about how and where the plant was collected, or what part of the plant went into the product. Such factors have a bearing on the content of potentially toxic compounds. Compounded by the commonly held belief that equates “natural” with health and well-being, the popularity of botanicals used for dietary supplements has dramatically increased in industrialized countries.

The occurrence of liver disease resulting from ingestion of herbal teas containing pyrrolizidine alkaloids have been known for some time. There is a longstanding tradition of medicinal herb use in Jamaica and the West Indies (Asprey and Thornton, 1955). In the 1950s, Bras and co-workers reported on endemic VOD in Jamaica (Bras et al., 1954). Liver biopsies, taken mostly from children, showed nonportal liver pathology typical of pyrrolizidine alkaloid ingestion with the key features of inflammation and veno-occlusion with intact hepatic portal triads, termed “nonportal” cirrhosis. More than 50% of the patients affected in Jamaican outbreaks fully recovered after sodium and fluid restriction. In the West Indies, acute VOD, which largely affects children, is associated with consumption of folk medicines made from Crotalaria fulva, which contains the pyrrolizidine alkaloid fulvine (Figure 3) (McLean, 1970). These medicines, or “bush teas,” which are hot-water infusions prepared from various plants, are generally bitter tasting, and are traditionally given to children as a tonic for ailments as diverse as a cold, teething pain and gastric problems.

A number of reports described clinically evident liver diseases resulting from either short- or long-term use of pyrrolizidine alkaloid-containing herbs. Probably the most popular and widely used pyrrolizidine alkaloid-containing medicinal herb is comfrey (Symphytum). Since Greek and Roman antiquity, this herb has been part of the official pharmacopeia of many cultures, and has been used as a “cure-all” for a variety of ailments and complaints. In fact, the species name officinale indicates its place in medieval herbs (Huxtable, 1989). Comfrey and Russian comfrey (S. uplandicum) are cultivated throughout Europe, North America and Australia.
The name comfrey may have been derived from the Latin *confirmare*, “to strengthen,” owing to its reputed ability to promote overall health and well-being, as well as heal fractured bones more quickly. Other uses for comfrey have included external use for wounds, and internal use for joint inflammation, gout, haematomas, gastritis, diarrhea, rheumatoid arthritis, bronchitis, backache and various allergies (Stickel and Seitz, 2000). Comfrey can also be found in a range of cosmetics and personal-care products, such as shampoo, skin creams, bath oils and various ointments.

Another popular form is a comfrey-pepsin capsule marketed as a digestive aid. According to Huxtable (1989), one preparation claiming to contain comfrey leaves contained 40 mg kg\(^{-1}\) pyrrolizidines and 230 mg kg\(^{-1}\) pyrrolizidine N-oxides, while another claiming to contain comfrey root had a total content of 2900 mg kg\(^{-1}\), consisting of 400 mg kg\(^{-1}\) pyrrolizidines and 2500 mg kg\(^{-1}\) of the N-oxides. As people can take several of these capsules per day, these products represent a major source of exposure to pyrrolizidines.

Comfrey contains a mixture of pyrrolizidine alkaloids, including intermedine, acetylintermedine, lycopsamine, acetyllycopsamine, symphytine, echimidine and symviridine, all of which are hepatotoxic (Huxtable, 1989). The overall alkaloid content of the plant varies from 0.003 to 0.2% for dry leaves and 0.2 to 0.4% for root (Roitman, 1981). These variations are mostly due to species, age of plant, location and season collected. Roitman found 8.5 mg of total alkaloids in a cup of comfrey root tea. Mattocks observed that because the leaf contains a relatively low concentration of alkaloids (which are largely N-oxides), and because the acute toxicity in rats of comfrey alkaloids is approximately half that of other pyrrolizidine alkaloids such as heliotrine (Culvenor *et al*., 1980), it is unlikely that people can be acutely poisoned by drinking comfrey tea (Mattocks, 1986). He predicted that a person weighing 60 kg would require 700 cups of comfrey tea (with 8 mg total alkaloids) to be so poisoned. However, poisoning by pyrrolizidine alkaloids is accumulative and generally chronic. Predictably, chronic comfrey use has been involved in a number of poisonings in people.

Ridker *et al*. (1985) reported hepatic VOD and centrilobular necrosis in a 49-year-old woman who was a “heavy consumer” of herbs, vitamins, and natural food supplements and a regular drinker of commercial comfrey tea. Additionally, she took six comfrey-pepsin capsules per day. From these sources, and from the regularity of use, the authors calculated that she had consumed 15 µg kg\(^{-1}\) day\(^{-1}\) or a minimum of 85 mg of pyrrolizidine alkaloids in the 4-months previous to her admission to hospital.

In another case, a 13-year-old boy was diagnosed with classic VOD who had for 2–3 years regularly consumed comfrey tea as a home treatment prescribed by a homeopath (Weston *et al*., 1987). The authors were con-
fident that comfrey ingestion was the only plausible explanation for this condition.

That regular use of comfrey products has serious health consequences was confirmed in a later clinical report of hepatic VOD diagnosed in a 47-year-old woman who was given a prescription of comfrey from a homeopathic doctor to cure abdominal pain, fatigue and allergies (Bach et al., 1989). The patient consumed as many as 10 cups of comfrey tea per day, as well as comfrey-pepsin capsules “by the handful.”

A fatal case of hepatic VOD associated with regular comfrey use was reported in a 23-year-old man who ate 4–5 steamed comfrey leaves 1–2 weeks before the onset of symptoms (Yeong et al., 1990). Other possible causes of VOD were excluded by these investigators. In another report, McDermott and Ridker described classic VOD associated with heavy comfrey use (McDermott and Ridker, 1990).

Other, less common, medicinal plants that contain pyrrolizidine alkaloids have been reported to cause poisonings. Daily consumption of the pyrrolizidine alkaloid-containing herbal medicines by a pregnant woman resulted in transmission of fatal VOD to her newborn infant (Roulet et al., 1988). The responsible pyrrolizidine alkaloid was identified as senecionine from the herb Tussilago farfara, which the mother purchased from a pharmacy. This herb, which is also known as coltsfoot, or the Old English designation coughwort, has been used since antiquity as a cough suppressant. Assuming a pyrrolizidine alkaloid content in the tea of 0.6 mg per kg dry weight, the authors calculated a cumulative transplacental exposure to the baby of approximately 0.125 mg total pyrrolizidine alkaloids per kg body weight. This was the first report of transplacental transmission of a pyrrolizidine alkaloid in people. It was later reported that the tea involved in this fatal case of poisoning also contained roots of Petasites officinalis (Spang, 1989).

Veno-occlusive disease was diagnosed in an 18-month-old boy who had regularly consumed a herbal tea mixture since the 3rd month of life (Sperl et al., 1995). The tea, given to the boy to promote “healthy development,” was gathered by the boy’s mother in her own garden. It consisted of peppermint leaves and Adenostyles alliariae or “Alpendost,” which the mother misidentified as coltsfoot (also likely to be toxic!). The two plants can easily be confused, especially after the flowering period. Analysis of the tea mixture revealed high amounts of seneciphylline (Figure 3) and its N-oxide. The authors calculated that the child had consumed at least 60 µg per kg body weight per day of pyrrolizidine alkaloids over 15 months. Fortunately, the child recovered completely within 2 months following conservative treatment.

Several wildflowers native to the southwestern United States and northern Mexico in common use as folk medicines have been shown to
contain pyrrolizidine alkaloids. There have been several poisoning incidents in which these plants are implicated. A 6-month-old girl regularly given home-brewed tea made from *Senecio longilobus* developed VOD which progressed over 2 months to extensive hepatic fibrosis (Stillman *et al*., 1977). Tea made from this plant had 3 mg g⁻¹ of pyrrolizidine alkaloids, and 10.5 mg g⁻¹ of *N*-oxides. By preparing a tea made according to the mother’s recipe, the authors calculated that during the 2 weeks before admission, the child received between 70 and 140 mg of pyrrolizidine alkaloids, clearly a toxic dose. Indigenous to the southwestern United States and northern Mexico, and known by Hispanics as “gordolobo yerba,” hot-water infusions of *Senecio longilobus* are used as a gargle and cough suppressant.

Another medicinal plant native to the southwestern United States, *Pedicularis*, also known as “betony,” “lousewort,” “Indian Warrior,” and “Elephant Head,” among other appellations, contains pyrrolizidine as well as toxic quinolizidine alkaloids through transfer by root parasitism from alkaloid-containing host plants, such as *Senecio triangularis* (Schneider and Stermitz, 1990). The major alkaloid found in this plant was seneconine. This is in stark contrast with the commonly held view that this plant is safe for use by children. For example, this plant was described in the “Bible” of herbal remedies entitled *Medicinal Plants of the Mountain West* (Moore, 1979) in this way:

> Betony is an effective sedative for children [my emphasis]. It acts as a mild relaxant … quieting anxieties and tension … Large quantities may cause a befuddled lethargy and some interference of motor control … It wouldn’t hurt to test a particular collection before administering freely, since the potency of various species is variable.

*Castellija* sp., or Indian paint brush, a native southwestern plant that has some traditional medicinal use, may contain pyrrolizidine alkaloids through root parasitism from another medicinal pyrrolizidine alkaloid-containing plant, *Liatris punctata* (Mead *et al*., 1992). Also known as “cachana,” “grey feather” and “blazing star,” *Liatris* is used as a diuretic, to treat throat inflammation and laryngitis, and as a cough suppressant. Burning *Liatris* root is inhaled as a cure for nosebleeds and tonsillitis (Moore, 1979). *Liatris punctata* was found to contain seneconine, intermedine and lycopsamine, in addition to a novel open-diester pyrrolizidine alkaloid, punctanecine (Mead *et al*., 1992). Another medicinal Indian paint brush, *C. rhexifolia*, contains pyrrolizidine alkaloids (Stermitz and Suess, 1978).

Seneconine and other hepatotoxic pyrrolizidine alkaloids and *N*-oxides (0.36% in aerial parts, 0.76% in roots) were found in *Packeria candidissima*, a Mexican medicinal herb commonly used to treat kidney ailments, ulcers,
and for its antiseptic properties (Bah et al., 1994). Among Mexicans in rural and urban areas of the state of Chihuahua and by Hispanics in southwestern United States, *Packeria* is commonly known as “chucaca,” “lechuguilla de la sierra,” “té de milagros” and “hierba de milagro.”

There are several other reports of fatal and non-fatal liver disease resulting from use of various pyrrolizidine alkaloid-containing herbal products (Lyford et al., 1976; McGee et al., 1976; Margalith et al., 1985; Jones and Taylor, 1989).

### V. TOXICITY OF PYRROLIZIDINE ALKALOIDS

Many pyrrolizidine alkaloids are carcinogenic in a number of animal models. (Schoental, 1968; Hirono, et al. 1973, 1978), although there are not sufficient data to conclude that they are carcinogenic in people. Much of the quantitative data on the toxicity of pyrrolizidine alkaloids are from shorter-term lethality studies. These data can give some indication about the relative toxicity of various pyrrolizidine alkaloids. There is a considerable difference in the toxicity among pyrrolizidine alkaloids (Table III). For example, the more potent alkaloids to which people are exposed, such as trichodesmine, senecionine and seneciphylline, have acute lethal toxicity (i.e. LD$_{50}$) values of 25, 50 and 77 mg kg$^{-1}$, respectively, while for heliotrine, lycopsamine and heliotrine N-oxide the LD$_{50}$ values are 296, >1000 and c. 5000, respectively (Table III). Monocrotaline is in the middle range of these toxins.

Similarly, there is a considerable range of susceptibility among animal species to pyrrolizidine alkaloids. Acute lethal toxicity of retrorsine, a

### TABLE III

**COMPARATIVE ACUTE TOXICITY OF SOME PYRROLIZIDINE ALKALOIDS**

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>LD$_{50}$ (mg kg$^{-1}$, i.p.)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichodesmine</td>
<td>25</td>
</tr>
<tr>
<td>Senecionine</td>
<td>50</td>
</tr>
<tr>
<td>Seneciphylline</td>
<td>77</td>
</tr>
<tr>
<td>Lastiocarpine</td>
<td>77</td>
</tr>
<tr>
<td>Monocrotaline</td>
<td>109</td>
</tr>
<tr>
<td>Heliotrine</td>
<td>296</td>
</tr>
<tr>
<td>Lycopsamine</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Heliotrine N-oxide</td>
<td>c. 5000</td>
</tr>
</tbody>
</table>

$^a$Male rats, intraperitoneal (i.p.) administration.
Adapted from Mattocks (1986) and references therein.
12-membered α,β-unsaturated pyrrolizidine alkaloid similar to senecionine, varies from 34 mg kg\(^{-1}\) in rats to 279 mg kg\(^{-1}\) in quail and over 800 mg kg\(^{-1}\) in guinea pigs (White \textit{et al.}, 1973). Like most other toxins, a major factor in species susceptibility to pyrrolizidine alkaloids is the ability of that animal to metabolize the parent compound into the active, electrophilic intermediate. Generally, susceptible animals form more of the reactive pyrrole than do resistant animals. Some evidence indicates that pyrrolizidine alkaloids may be degraded and hence detoxified by rumen microflora in resistant species.

Postulated as a possible etiology of Indian childhood cirrhosis, which is characterized by excessive hepatic copper accumulation (Morris \textit{et al.}, 1994; Aston \textit{et al.}, 1996), copper and pyrrolizidine alkaloids have been shown to exhibit true hepatotoxic synergism in a number of animal models. In rats, retrorsine and copper given concurrently result in significantly greater mortality, liver pathology and hepatic copper accumulation compared to that seen when either of these agents was administered alone (Morris \textit{et al.}, 1994). Likewise, heliotrope hepatotoxicity in sheep was markedly enhanced by concurrent or subsequent administration of copper (Howell \textit{et al.}, 1991). Those authors noted that hepatic copper concentrations were higher in sheep given heliotrope + copper compared to those given the same amount of copper alone.

Serum copper concentrations have been used as a noninvasive indicator for monocrotaline-induced cardiopulmonary toxicity (Molteni \textit{et al.}, 1988). Retrorsine passing to rat neonates via breast milk results in the accumulation of hepatic copper, an impairment of the rise in serum ceruloplasmin, and a decrease in hepatic metallothionein and serum albumin levels (Aston \textit{et al.}, 1996). The authors suggested that accumulation of liver copper and reduction of copper-binding proteins could result in an increase of free copper that might enter into pro-oxidant Fenton-type reactions, resulting in the generation of oxygen free radicals. The mechanism of the synergy between copper and pyrrolizidine alkaloids probably centers around the fact that both compete for the same pool of cellular glutathione (GSH). Copper uptake and incorporation into metallothionein is intimately associated with reduced GSH, and GSH is generally thought to act as a protectant against oxidant damage (Freedman \textit{et al.}, 1989). In fact, depletion of GSH potentiates metal toxicity in a variety of animals and cell systems (Freedman \textit{et al.}, 1989). Similarly, GSH depletion resulted in an increase of the release of active pyrroles from rat liver, which are then available for macromolecular alkylation and toxicity (Yan and Huxtable, 1995).

As a group, pyrrolizidine alkaloids are hepatotoxic, but depending on the alkaloid, other extra-hepatic effects may result from ingestion. One important distinction of pyrrolizidine alkaloid poisoning is that it is
progressive, once it has been initiated by exposure to even a single moderate dose. The most prevalent pyrrolizidine alkaloid-caused diseases in people are hepatic VOD, pulmonary hypertension and cor pulmonale, or congestive right heart failure (Huxtable, 1990). Hepatotoxicity commonly results from pyrrolizidine alkaloids found in *Senecio* species, such as senecionine, retrorsine, seneciphylline and riddelliine, while cardiopulmonary toxicity is more likely to result from *Crotalaria* alkaloids, such as monocrotaline. Trichodesmine, an alkaloid very similar to monocrotaline (Figure 2), is a neurotoxin (Yan *et al.*, 1995).

In people, the most common clinical sequela is VOD caused by an occlusion of the smaller branches of the hepatic vein due to endothelial proliferation and medial hypertrophy (Huxtable, 1989). This occlusion leads to centrilobular congestion and a pooling of blood. Clinically, VOD can be divided into an acute, a subacute and a chronic phase.

The acute phase is characterized by a rapid onset of nausea, emesis, abdominal pain, distension, portal hypertension, reduced urinary output, hepatomegaly and ascites (Stillman *et al.*, 1977; Sperl *et al.*, 1995). In this phase, poisoning victims may either expire, recover completely (especially if pyrrolizidine alkaloid ingestion is discontinued), or progress to the subchronic and chronic phases (Stillman *et al.*, 1977). The subchronic phase involves persistent hepatomegaly and recurrent ascites. The chronic phase consists of cirrhosis and liver failure, and may be delayed for months or years following exposure. There is no specific antidote for pyrrolizidine alkaloid poisoning, except supportive treatment.

Aside from being occasionally seen as a result of chemotherapy and bone marrow transplantation, VOD is diagnostic for pyrrolizidine alkaloid poisoning. It has a poor long-term prognosis, and death may occur anywhere from 2 weeks to over 2 years following exposure. Children are more vulnerable than adults to VOD.

A small number of pyrrolizidine alkaloids, of which monocrotaline is the best example, result in cardiopulmonary toxicity. The pathology and pulmonary toxicology of monocrotaline is well studied. As with other pyrrolizidine alkaloids, the toxicity depends upon hepatic metabolic activation, which, in this case, results in the formation of monocrotaline pyrrole, or dehydromonocrotaline. Unlike hepatotoxic pyrrolizidine alkaloids such as senecionine, monocrotaline exerts its primary toxicity in an organ distant to the location of metabolic activation. The lung appears not to activate monocrotaline to any appreciable extent. Monocrotaline had no effect when perfused through isolated rat lungs, but induced pulmonary toxicity when first perfused through isolated liver (Lafranconi and Huxtable, 1984). The selectivity of monocrotaline for the lung is dependent upon the binding of the monocrotaline pyrrole by red blood
cells, where it is stabilized during transport to the lung (Wilson et al., 1992). Toxicity to the lung proceeds through an inflammatory response, for which platelet activation plays a role. In animals, this results in pulmonary hypertension in which an abrupt elevation in pulmonary arterial pressure occurs, and acute cor pulmonale. This pathology, while not known to occur in people exposed to monocrotaline, closely follows acute respiratory distress syndrome (ARDS), a syndrome known to be caused by a variety of environmental agents.

As mentioned earlier, diagnosis pyrrolizidine alkaloid poisoning is often difficult, especially when the disease takes a chronic course. There are several factors that confound a correct diagnosis. These include the varying interval between ingestion and development of symptoms, and that health care providers may not query the patient about herbal supplement use. Unfortunately, there is little general appreciation that natural “organic” supplements and tonics may indeed be harmful. Furthermore, pyrrolizidine alkaloid-related diseases can easily be confused with other pathologies. For example, chronic hepatic cirrhosis induced by pyrrolizidine alkaloids is clinically indistinguishable from that caused by alcohol, infection, or any number of other etiologies. Veno-occlusive disease has been confused with viral hepatitis (Datta et al., 1978a). Pyrrolizidine intoxication may also have a few symptoms in common with Reye’s syndrome, as was documented in the fatal poisoning case of a 2-year-old child (Fox et al., 1978).

Despite the fact that pyrrolizidine alkaloids are known to be carcinogenic in animals, and are genotoxic in a variety of short-term in vitro and in vivo systems, there is insufficient evidence to conclude that long-term exposure to pyrrolizidine alkaloids causes cancer in people.

VI. METABOLISM OF PYRROLIZIDINE ALKALOIDS

Like thousands of other “pro-toxicants” such as aflatoxin B$_1$, benzo[a]pyrene, and dimethylnitrosamine, pyrrolizidine alkaloids are not toxic per se, but must first be metabolized by endogenous enzymes following ingestion of the plant material. Several classes of enzymes act on pyrrolizidine alkaloids. In mammals, the most important examples are the cytochromes P450 (CYP). Cytochromes P450 are a large group of hemoprotein enzymes that are present in greatest quantities in the liver, but are also found in the lung, kidney, brain and other organs. CYPs utilize reduced NADPH + H$^+$ as a cofactor together with NADPH cytochrome P450 reductase to reduce active site iron to the ferrous form to catalyze insertion of oxygen into the substrate. Hundreds of CYP isoforms have been isolated, each having various substrate specificities.
Metabolic conversion of pyrrolizidine alkaloids results in several possible products with varying degrees of toxicity. The three main phase I enzymatic reactions acting on pyrrolizidine alkaloids in the liver are: hydrolysis, resulting in the formation of a free necic acid and necic base; N-oxidation, to form the N-oxides, which are of modest toxicity (this pathway is therefore considered a detoxification); and dehydrogenation to form reactive and toxic pyrroles, also called dehydroalkaloids (Figure 4). As with many other proximal, reactive intermediates (such as the aflatoxin 8,9-epoxide, and benzo[a]pyrene diol-epoxide), pyrroles are electrophilic and inherently unstable, and react quickly with endogenous nucleophilic macromolecules.

The current hypothesis for the mechanism for production of the pyrrolic intermediate involves an initial CYP-mediated hydroxylation of the pyrrolizidine alkaloid at C-8, producing a chemically unstable carbinolamine, which spontaneously loses the hydroxyl group, then a proton, giving the dehydropyrrolizidine (Mattocks, 1986; Rajski and Williams, 1998) (Figure 5). There are several fates for these reactive pyrroles, which have a half-life of a few seconds.

In liver tissue from people and rodents, CYP 3A4 is an important isoform catalyzing dehydrogenation. This isoform is the most prevalent in human liver (approximately 60% of total), and is inducible by compounds such as barbiturates, dexamethazone and erythromycin (Guengerich, 1989). This enzyme has broad substrate specificity; indeed, approximately half of the drugs currently on the market are substrates for CYP 3A4 (Ueng et al., 1997). The prototype activity of CYP 3A4, which is the basis of diagnosing 3A4 activity of various tissues, is the oxidative conversion of the calcium channel blocker nifedipine (NF) to its main metabolite, dehydronifedipine. Good evidence of the critical role of CYP 3A4 in pyrrolizidine alkaloid activation was provided by the observation that two mechanism-based inhibitors of human liver CYP 3A4, gestodene and triacetyloleandomycin, inhibited the conversion of senecionine to dehydroseconicine and senecionine N-oxide in human liver microsomes (Miranda et al., 1991).

N-Oxidation of pyrrolizidine alkaloids appears to be catalyzed by CYP 2C11 and to a lesser extent by CYP 3A. In hepatic microsomal preparations from guinea pig, N-oxide formation is also catalyzed by flavin-containing monooxygenases (FMO) (Williams et al., 1989). The formation of the toxic pyrroles and detoxified N-oxides occurs by different pathways. This conclusion has been reached largely from the observation that plant N-oxides are not activated to pyrroles in microsomal preparations in vitro. The actual mechanism of N-oxide formation has not been elucidated.
An important route of detoxification is mediated by the universal detoxifying enzyme, glutathione S-transferase (GST), to produce various glutathione (GSH) conjugates that are water soluble and hence more easily excretable than the parent compound (Figure 4). Glutathione S-transferases are a group of cytosolic homo- and heterodimeric proteins that catalyze the detoxification of a large number of compounds which, like pyrrolizidine alkaloids, are metabolized to electrophilic intermediates (Boyer, 1989). Like the CYPs, the GSTs are a multienzyme family with varying substrate specificities. Which GST isoforms are involved in catalysis of pyrrolic detoxification has not yet been elucidated. Various GSH conjugates were frequently the major detoxified products of pyrrolizidine alkaloids in isolated, perfused rat liver (Yan et al., 1995). Activated pyrroles may polymerize, resulting in detoxified products.

A variety of cellular nucleophiles are known to react with pyrrolic pyrrolizidine alkaloids and hence may be involved in detoxifying Sn1-type reactions. The thiols GSH and cysteine (Robertson et al., 1977), and thiol resins (Glowaz et al., 1992) efficiently react with pyrroles. Dietary cysteine resulted in protection against pyrrolizidine alkaloid-induced hepatotoxicity compared to control rats without cysteine (Miranda et al., 1982). Glutathione and cysteine, but not methionine, competed with λ-phage DNA for pyrrolic cross-linking, indicating that thiols with a free sulfhydryl group are sufficiently reactive to compete with DNA for reaction with pyrroles (Coulombe et al., 1999).

VII. MECHANISM OF TOXIC ACTION

Enzymatic oxidation of pyrrolizidine alkaloids to the pyrrole results in the production of a bifunctional intermediate with reactive, electrophilic centers at C-7 and C-9 by conjugation of the pyrrole nitrogen lone pair. These electrophilic centers react with a variety of nucleophilic cellular macromolecules, possibly the most critical of which are various nucleoside bases in DNA, resulting in the formation of cross-links between two strands of DNA (DNA–DNA cross-links) or between one strand of DNA and some cellular protein (DNA–protein cross-links; DPCs) (Figure 5). It has been postulated that nucleophilic substitution at C-7 is favored over C-9 owing to greater stabilization of the secondary carbonium ion at C-7 (Huxtable and Cooper, 2000). In cultured cells, various pyrrolic pyrrolizidine alkaloids form an approximately equal proportion of these cross-links, and are inherently DNA repair-resistant (Kim et al., 1993, 1995).

A number of reports strongly support the hypothesis that the formation of DNA cross-links is an important molecular event in the toxicity of
Senecionine

\[
\text{N} \quad \text{O} \quad \text{O} \\
\text{H} \quad \text{H} \quad \text{H}
\]

hydrolysis

CYP 3A, 2C11, FMO (N-oxidation)

Senecic acid

Dehydrosenecionine

CYP 3A4-mediated oxidation

Dehydroretronecine react with tissue nucleophiles (see Figure 5)

glutathione conjugate

GSH/GST

Retronecine

[O]

H

O

H

Dehydrosenecionine

GSH/GST

R. A. COULOMBE
FIG. 4. Metabolic fate of a representative pyrrolizidine alkaloid senecionine showing the various phase I and phase II reactions. Alkaloids are metabolized by a variety of enzymes, the most important of which are the cytochromes P450 (CYP). Activated pyrrolic intermediates may react with glutathione (GSH) under catalysis of glutathione S-transferase (GST), resulting in a water-soluble conjugate that is easily excreted. Pyrrolic intermediates may also form cross-links with tissue nucleophiles, such as DNA and proteins, resulting in toxicity. Alternatively, the pyrrole may be detoxified by CYP or flavin monooxygenases (FMO) to N-oxides, or hydrolyzed to retronecine and senecic acid.
FIG. 5. Postulated sequential mechanism of cytochrome P450-(CYP)-mediated oxidative activation of senecionine, and formation of a cross-link. Generation of the pyrrolic intermediate results in activation to electrophilic centers at the C-7 and C-9 by conjugation of the pyrrole nitrogen lone pair. Once formed, the activated, bifunctional pyrrolic intermediate can form cross-links with cellular nucleophiles (Nu), such as DNA, and protein, such as actin. It has been postulated that nucleophilic substitution at C-7 is favored over C-9 owing to greater stabilization of the secondary carbonium ion at C-7. Adapted from Huxtable and Cooper (2000) and Rajsik and Williams (1998).
pyrrolizidine alkaloids. For example, the degree to which various pyrrolic pyrrolizidine alkaloids cause cytotoxic end points such as megalocyte formation and inhibition of colony formation correlates with the formation of DNA cross-links in cultured mammalian cells (Kim et al., 1993). Megalocytosis is seen in the liver of animals exposed to pyrrolizidine alkaloids. It is a condition where cells have an unusually large cellular and nuclear volume, due to the antimitotic effect of the pyrrolizidine alkaloids where biosynthesis of cellular precursors occur, but without formation of mitotic spindles and mitosis (Figure 6). Another report demonstrated that DNA cross-link formation by pyrrolizidine alkaloids interferes with DNA replication. Dehydrosenecionine (DHSN) and dehydromonocrotaline (DHMO) interrupt the polymerase chain reaction amplification of a segment of pBR322 (Kim et al., 1999). This implies that cross-linking by activated pyrrolizidine alkaloids is functionally significant in the cell.

There is a significant difference in cross-link potency among pyrrolizidine alkaloids. In a series of structure–activity studies, it was found that structural features, most notably the presence of $\alpha,\beta$-unsaturation and a macrocyclic diester, confer potent cross-link and megalocytic activity to pyrrolizidine alkaloids in cultured bovine kidney (MDBK) cells (Kim et al., 1993, 1999). In this system, macrocyclic pyrrolizidine alkaloids with $\alpha,\beta$-unsaturation (seneciphylline, senecionine, riddelliine and retrorsine) were significantly more potent DNA cross-linkers and inducers of megalocytosis than was the $\alpha,\beta$-saturated monocrotaline. The open diester pyrrolizidine alkaloids (latifoline and heliosupine) were less potent than monocrotaline, and the simple neine base retronecine was the least potent of any of these. Ironically, indicine N-oxide, which had demonstrated activity against acute leukemia in clinical trials, but was discontinued because of myelosupression and severe hepatotoxicity in human subjects (King et al., 1987), did not induce any measurable DNA cross-links (Kim et al., 1993).

The rank order of relative DNA cross-link potencies is often reflected in the animal toxicity of these pyrrolizidine alkaloids. For example, the acute toxicities in rats of senecionine, seneciphylline and retrorsine are approximately equal, but significantly higher than that of monocrotaline (Huxtable and Cooper, 2000).

Chemically reactive pyrrolic pyrrolizidine alkaloids share a common pyrrolic substructure with reductively activated bifunctional mitomycins, such as mitomycin C, which preferentially cross-link 5′-CG sequences within DNA (Woo et al., 1993). There is conflicting evidence as to whether activated pyrrolizidine alkaloids share similar cross-linking base or DNA sequence specificity. Several DNA bases have been shown to be involved in covalent interactions and/or cross-links by pyrrolic pyrrolizidine alkaloids. For example, dehydroretronecine reacts with purine and pyrimidine
nucleosides such as the N² of deoxyguanosine (dG) and N⁶ of deoxyadenosine (dA). The O² sites of uridine and deoxythymidine or thymidine (dT) have also been identified as targets (Robertson, 1982; Wickramanayake et al., 1985). Dehydromonocrotaline and dehydroretorsine preferentially cross-
linked dG-to-dG at a 5′-CG sequence in synthetic duplex DNA (Weidner et al., 1990). Dehydromonocrotaline was shown to cross-link at the N-7 position of guanine in a 35 bp fragment of pBR322 with a preference for 5′-GG and 5′-GA sequences. However, because these authors examined only alkylation at guanyl residues, no light was shed on whether DHMO alkylated at other sites (Pereira et al., 1998).

Our laboratory has recently shown that the pyrrolic pyrrolizidine alkaloids dehydrosenecionine, dehydroretrorsine and dehydromonocrotaline had little, if any, discernible sequence specificity when cross-linked to a series of synthetic 32P end-labeled oligonucleotides of varying DNA sequence (Coulombe and Rieben, 2002). These oligonucleotide targets were 14- or 24-base poly dA-T that had different central base sequences such as 5′-d(CG), 5′-d(GC), 5′-d(TA); 5′-d(CGA), 5′-d(GCA) or 5′-d(TAA). Similar model oligonucleotides have been used to determine possible sequence recognition of other bifunctional electrophiles, such as mitomycin C (Weidner et al., 1990).

Less is known about the characteristics of the pyrrolizidine alkaloid-induced DNA–protein cross-link. Initial studies from our laboratory showed that a major protein released by DNAse I treatment of DNA–protein complexes purified from pyrrolizidine alkaloid-treated bovine kidney cell (MDBK) nuclei had a molecular weight of c. 43 kD, was acidic (pI ≈ 4.5) and had a two-dimensional electrophoretic pattern similar to that of cells treated with cisplatinum, a benchmark bifunctional cross-linker known to cross-link DNA with actin (Kim et al., 1995). A follow-up study using an anti-actin multiple antigen peptide (MAP) antibody confirmed that actin is the major protein involved in pyrrolizidine alkaloid-induced DNA–protein cross-links in human breast carcinoma (MCF-7) and in Madin–Darby bovine kidney (MDBK) cells (Coulombe et al., 1999). The cross-link pattern by two pyrrolic pyrrolizidine alkaloids dehydrosenecionine and dehydromonocrotaline was similar to that of cisplatinum as well as mitomycin C, a pyrrolic anti-cancer drug, although these benchmark compounds were more potent cross-linkers than the pyrrolizidine alkaloids (Figure 7).

The involvement of actin in pyrrolizidine alkaloid-induced DPCs is a reasonable expectation due to the abundance of this protein in the nuclear matrix. Actin is also a target for cross-linkers such as cisplatinum and trivalent chromium (Miller et al., 1991), and mitomycin C (Coulombe et al., 1999). Inasmuch as the megalocytic and antimitotic effects of pyrrolizidine alkaloids coincide with cross-linking potency (Kim et al., 1993), it is plausible that the antimitotic action of pyrrolic pyrrolizidine alkaloids in vitro and in vivo may be explained, at least in part, by their ability to cross-link DNA with actin. In addition to actin, other protein
targets that were later identified in dehyrdomonocrotaline-treated cultured pulmonary artery endothelial cells included galectin-1 and protein-disulfide isomerase (Lame et al., 2000). The functional significance of a monocrotaline cross-link to these other proteins is not known.

VIII. CONTROL OF PYRROLIZIDINE ALKALOIDS AND FUTURE PROSPECTS

Usual practices of treating cereal crops with herbicides to reduce weed infestation and subsequent quality assurance steps such as grain inspection can help prevent large-scale outbreaks of poisoning due to contamination of pyrrolizidine alkaloid-containing seeds or plants in staple food. Modern winnowing techniques readily separate toxic Heliotropium, Senecio and Crotalaria seeds from cereal grain, at least to acceptable tolerances. However, it is likely that drought, famine and political instability will again conspire to produce another large-scale outbreak of human poisonings.

Periodic exposure to small amounts of pyrrolizidine alkaloids in foods such as milk, honey and eggs is also likely to occur. University-based outreach and information programs may be one mechanism by which producers can become better educated on ways to minimize potential pyrrolizidine alkaloid contamination in their products. For example, honey producers may benefit from learning if plants their bees are likely to pollenate contain pyrrolizidine alkaloids. They can then move their hives to other fields, thereby preventing or reducing contamination.

The many poisoning cases resulting from consumption of “safe” and “natural” supplements emphasizes the urgent need for greater awareness of the potential adverse health effects of herbal products. Years of traditional use of a botanical supplement is no guarantee of safety. This caveat is especially true in the case of pyrrolizidine alkaloids and other chemicals, whose effects can be delayed months or even years. Such chronic toxicoses are notoriously difficult to diagnose.

Public health campaigns against the use of Crotalaria fulva have been very effective in reducing VOD in Jamaica (Mattocks, 1986). In the United States, unfortunately, much of the popular literature on herbal supplements, as well as nearly all of the product “fact sheets” provided by health food stores (produced by the companies that produce the herb products), are misleading. In the case of pyrrolizidine alkaloid-containing plants like comfrey and coltsfoot, new legislation is needed to protect consumers.

Clearly, existing laws, such as the Dietary Supplement Health and Education Act Congress, need to be significantly modified in the interest of consumer protection. In an article entitled “Herbal Roulette,” Consumer’s
FIG. 7. Western immunoblots showing the presence of actin isolated from proteins released from purified DNA–protein cross-links in Madin–Darby bovine kidney (MDBK; left) and human breast carcinoma MCF-7 (right) nuclei treated with DMSO vehicle (control), and the pyrrolic pyrrolizidine alkaloids dehydrosenecionine (DHSN), or dehydromonocrotaline (DHMO). Anti-cancer cross-linking agents mitomycin C (MMC), or cisplatinum are included for reference. Released proteins that were originally cross-linked to 40 µg nuclear DNA were loaded onto SDS-PAGE (11% running, 4.5% stacking) gels, electrophoresed, transferred to membranes, then probed with a monoclonal anti-actin multiple antigen peptide (MAP) antibody that recognizes an epitope conserved in all actin isoforms. α-Skeletal actin was used as a standard (α-actin). Relative densitometric intensities of actin signals were normalized to that observed from cisplatinum-induced DNA–protein cross-links. Reproduced from Coulombe et al. (1999) by permission.

Union recommended that changes to the law should include: clearer disclaimers, in large type, stating that any claims of safety and efficacy are strictly the claims of the manufacturer, and have not been confirmed by the FDA; consistent manufacturing and content standards; and banning clearly dangerous supplements (Consumer’s Union, 1995). Another worthwhile step would be to adapt the approach of the German Commission E, which was established to evaluate the safety and efficacy of herbal supplements on the basis of clinical trials and comprehensive risk assessment analyses. The Commission has published more than 320 monographs on herbs
(Klepser and Klepser, 1999). In the absence of these steps, people in the United States will continue to receive misinformation about the usefulness and safety of their “health” foods. In any event, an industry and government-sponsored campaign to increase awareness among herbalists, natural food proprietors, and Native American shamans on the deadly potential of their products will help. A few high-profile product liability cases may be the only impetus to motivate the industry to self-regulate.

Medical providers and users of herbs should be aware that young people are especially susceptible to the toxic effects of pyrrolizidine alkaloid-containing supplements. Medical providers should also query their patients on their possible use of home remedies and herbal supplements, especially when there are unexplained symptoms. An increased recognition of the relatively uncommon symptoms of pyrrolizidine alkaloid poisoning by the medical community will also increase the accuracy of incident reporting.

ACKNOWLEDGEMENTS

I wish to acknowledge generous support from NIH-NIEHS (ES04813), USDA-NRI (02-2780, 98-3754, 97-3081, AS-79), the Willard L. Eccles Foundation, the Dr W. C. Swanson Family Foundation, and from the Utah Agricultural Experiment Station, where this is designated as number 7452. This review is dedicated to Centennial Professor Frank R. Stermitz of Colorado State University – natural product chemist, teacher, mentor and friend.

REFERENCES


ULTRASONIC SENSORS FOR THE FOOD INDUSTRY

JOHN N. COUPLAND AND RAFFAELLA SAGGIN

Department of Food Science
The Pennsylvania State University
University Park, PA 16802–2504
USA

I. Introduction
II. Theory
   A. Ultrasonic Waves
   B. Ultrasonic Propagation in Homogeneous Materials
   C. Ultrasonic Propagation in Inhomogeneous Media
   D. Waves at Interfaces
   E. Diffraction
III. Measurement Methods
   A. Pulsed Modes
   B. Noncontact Measurements
   C. Resonance Methods
   D. Reflectance Methods
   E. Love Wave Sensors
IV. Applications
   A. Level Sensors
   B. Fluid Flow
   C. Temperature
   D. Composition
   E. Phase Transitions
   F. Rheological Properties
   G. Dispersed Systems and Food Microstructure
   H. Polymeric Systems
   I. Miscellaneous Applications
   J. Imaging
V. Conclusions
Acknowledgements
References
I. INTRODUCTION

Sensing and measurement of food properties are crucial to improving the quality and profitability of food manufacturing operations. Instrumental measurements can reduce the dependence on time-consuming chemical and sensory analysis. The ideal would be to make an assessment of the food quality as close to the processing step as possible. The measurement should provide some information about the food (e.g. temperature, composition, structure, concentration) useful in controlling the final product quality. The response time is crucial, so while laboratory tests on finished product are valuable, measurements, made at-line or on-line of the freshly made or in-process food are better. On-line sensors in the food industry must also be adequately cheap and robust to survive in the frequently hot and wet environment of a food processing plant. They should also be both nondestructive of the food products and amenable to hygienic design principles (ideally noninvasive) and provide output usefully to an operator or automated control system.

Sensing modalities meeting some or all of these conditions have found specific applications in the food industry (e.g., microwaves for water content, thermocouples for temperature, nuclear magnetic resonance (NMR) for solid fat content, and infrared (IR) for composition). Ultrasound has also been used, but to a much lesser extent. The different physical principles sensed ultrasonically provide a unique set of advantages (and disadvantages) as a food sensor.

Ultrasound has become invaluable as a nondestructive sensor for materials as diverse and valuable as a human fetus and an aircraft wing. The same principles that enable us to “see” these structures with sound have also allowed researchers to attempt to characterize foods ultrasonically. In many ways food is a more difficult material for nondestructive evaluation as low unit costs and high production rates make it uneconomic to have a trained technician make a piece-by-piece evaluation, and the sensor must be directly incorporated into the process.

There have been some important practical successes (e.g. ultrasonic flowmeters, carcass evaluation, concentration sensing and particle size determination), but a far wider set of studies that have never progressed beyond a few academic publications. One crude distinction between the successes and failures is that the physics of sound–material interactions is frequently underexploited by food scientists; another is that the complexities of food manufacture are often underestimated by nondestructive evaluation specialists. One of the goals of this chapter is to clearly set out some of the underlying physical principles and then, in a review of some applications, stress how they can lead to useful measurement.
We start by discussing the physics of sound and the interactions of sound with matter. We then describe the practicalities of ultrasonic measurement, and finally review some applications of ultrasonic measurements to foods, grouping them by the type of parameter measured rather than by food group. The field of ultrasonic evaluation of foods has been previously reviewed elsewhere, and the works of Povey (1997; Povey and Mason, 1998), McClements (1997), Javanaud (1988) and Kress-Rogers (1993) are all recommended. The bibliography provided by Povey (1998a) is particularly comprehensive.

II. THEORY

A. ULTRASONIC WAVES

A wave is a collective phenomenon, implying coherent local variation of elements of the structure. In an acoustic wave, the variation is in the position of volume elements of the media through which the sound is passing, and so the acoustic properties of a material depend on both its density (the amount of material to be moved) and mechanical properties (how easy it is to move). The strains within the material caused by the passing sound wave are coupled with other parameters including local energy, pressure, density and temperature of the medium. The rate of the temperature fluctuations is usually more rapid than heat flux; therefore, most theories of sound rely on the adiabatic assumption (i.e. no heat flux) (Strutt, 1945; Wood, 1955).

Ultrasound is qualitatively similar to audible sound except that the vibrations occur at frequencies (> 20 kHz) too high to be detected by the human ear. Indeed, a sound wave is part of the whole spectrum of material periodic motion (not including translational motion) that at very high frequencies is seen as thermal waves. Just as classical electromagnetic spectroscopy can probe different structures in the same material by interaction with light of different frequencies, ultrasonic spectra can in principle reveal detail at different levels of material structure. A further interesting parallel between electromagnetic and mechanical waves is that very high frequency sound is better thought of as a particle – a phonon – and the paradoxes of wave–particle duality are relevant; see Povey (1997) for further discussion.

The material oscillations associated with an acoustic wave can occur both normal to and parallel with the direction of transmission. When ultrasonic waves are transmitted through an elastic, inert material, each element will experience forces due to the fluctuating pressure and will
tend to move from its equilibrium position. For a sinusoidally varying pressure, the motion of the particle will also be sinusoidal and, depending on the balance of forces acting, will follow an elliptical path about its equilibrium position (Wood, 1955). The frequency of the motion is the same as the frequency of the sound and the amplitude of the motion is related to both the magnitude of the wave and the mechanical properties of the material. In most sensing applications, the acoustic power levels and hence the material movements are small and within the elastic limit of the material. It is therefore assumed that as the wave passes the material will return to its unperturbed state and will be physically and chemically unchanged (i.e. ultrasonic testing is nondestructive).

The displacement ($\xi$) of a particle in a periodic wave from its equilibrium position ($x$) with time ($t$) is given by:

$$\xi = a \sin 2\pi \left( vt - \frac{x}{\lambda} \right)$$

where $v$ is the frequency of the oscillation, and $a$ and $\lambda$ are the amplitude and wavelength of the sound. Eqn (1) can be formulated in terms of wave velocity ($c = v\lambda$) so the microscopic motion of volume elements of the material is described solely by the bulk properties of the wave.

Although sound can propagate by the oscillation of volume elements in three dimensions, ultrasonic measurements are often conducted using waves to induce motion in simpler paths. When an oscillating stress is applied perpendicular to the surface of a material, vibrations take place as a series of compressions and expansions in the direction of sound propagation (particles oscillate back and forth) and longitudinal or compression waves are generated. When the oscillating force is applied parallel to the surface of a material, the vibrations take place perpendicular to the direction of wave propagation and shear or transverse waves are generated (Figure 1). Shear waves can be generated by a shear transducer or by mode conversion of longitudinal waves at an interface (see section D below). The different types of wave are sensitive to different material properties but, as longitudinal waves have been predominantly used in food systems, we will, unless noted, restrict our discussion to this mode.

Longitudinal and shear waves are both bulk waves but it is also possible to generate waves trapped in a surface (which are often useful for detecting surface defects or composition). These waves are produced when longitudinal waves are directed at a critical angle (see section D below) so the refracted energy passes into the surface (Raichel, 2000). Two types of surface waves are Love waves, which are shear waves polarized in the plane of the surface, and Raleigh waves, which contain longitudinal and
shear components (Blitz, 1963). Surface waves can travel long distances and are sometimes used to detect flaws in pipes.

B. ULTRASONIC PROPAGATION IN HOMOGENEOUS MATERIALS

A material responds to the passing ultrasonic wave both elastically and viscously. These two phenomena can be expressed together as a complex wavenumber, $k$:

$$k = \omega/c + i\alpha$$  \hspace{1cm} (2)

where $\omega$ is the angular frequency ($= 2\pi v$), $\alpha$ is the attenuation coefficient and $i$ is $\sqrt{-1}$. Velocity is the distance traveled by the sound wave in unit time or the product of wavelength and frequency. Attenuation describes the logarithmic decrease in wave energy with distance ($x$), i.e.:
\[ A = A_0 \exp(-\alpha x) \quad (3) \]

where \( A_0 \) and \( A \) are the amplitudes at distance 0 and \( x \). The attenuation coefficient \( \alpha \) is measured in Nepers (Np) or decibels (dB) per meter (1 Np = 8.686 dB).

The propagation of sound through a material depends on the physical properties of that material:

\[ \left( \frac{k}{\omega} \right)^2 = \frac{\rho}{E} \quad (4) \]

where \( \rho \) is the density and \( E \) an appropriate elastic modulus. For materials where the attenuation is not large (i.e. \( \alpha \ll \omega/c \)), the imaginary component of the parameters described in Eqn (4) can be neglected and the expression can be rewritten as:

\[ c^2 = \frac{E}{\rho} \quad (5) \]

The elastic modulus used here is distinct from the elastic modulus used in ordinary static measurements. In static measurements, deformation is slow and the material is at thermal equilibrium prior to measurement, so it is the isothermal elastic modulus that is measured. However, when ultrasonic waves travel through a material, the deformations are extremely rapid and the local temperature rises during the compression phase of the cycle and decreases during rarefactions. Since the rate of pressure change is rapid, no heat exchange occurs between the hot and cold regions and ultrasonic propagation is dependent on the adiabatic elastic modulus.

Frequently we shall be concerned with measurements of either the real (velocity) or imaginary (attenuation) part of the wavenumber, but Eqn (4) is a useful reminder that these are parts of a single phenomenon. Similarly the complex parts of the other measured parameters can give independent information on material properties. The ultrasonic properties of selected materials are listed in Table I.

1. Ultrasonic velocity

Gases are able to support only longitudinal waves and in practice even these can be hard to measure (see below). For ideal gases the appropriate adiabatic elastic modulus is the bulk elastic modulus \( K \), equal to the product of the hydrostatic pressure \( P \) and \( \gamma \) (= \( C_p/C_v \), where \( C_p \) and \( C_v \) are the specific heats at constant pressure and volume). Depending on the
## TABLE I
ULTRASONIC PROPERTIES OF SELECTED MATERIALS. UNLESS NOTED, MEASUREMENTS WERE CONDUCTED AT 20°C

<table>
<thead>
<tr>
<th>Material</th>
<th>Frequency (MHz)</th>
<th>Ultrasonic velocity (m s(^{-1}))</th>
<th>Ultrasonic impedance ((10^6 \text{ kg m}^2 \text{ s}^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Longitudinal Shear</td>
<td>Longitudinal</td>
<td></td>
</tr>
<tr>
<td><strong>Non-food materials</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aluminum</td>
<td>6320</td>
<td>3130</td>
<td>17</td>
<td>Birks and Green (1991)</td>
</tr>
<tr>
<td>Brass</td>
<td>4400</td>
<td>2200</td>
<td>37</td>
<td>Birks and Green (1991)</td>
</tr>
<tr>
<td>Epoxy resin</td>
<td>2400–2900</td>
<td>1100</td>
<td>2.7–3.6</td>
<td>Birks and Green (1991)</td>
</tr>
<tr>
<td>Gold</td>
<td>3240</td>
<td>1200</td>
<td>63</td>
<td>Birks and Green (1991)</td>
</tr>
<tr>
<td>Perspex</td>
<td>2730</td>
<td>1430</td>
<td>3.2</td>
<td>Birks and Green (1991)</td>
</tr>
<tr>
<td>Plexiglas</td>
<td>2670</td>
<td>1120</td>
<td>3.2</td>
<td>Birks and Green (1991)</td>
</tr>
<tr>
<td>Quartz glass</td>
<td>5570</td>
<td>3520</td>
<td>14.5</td>
<td>Birks and Green (1991)</td>
</tr>
<tr>
<td>Rubber (soft)</td>
<td>1480</td>
<td>–</td>
<td>1.4</td>
<td>Birks and Green (1991)</td>
</tr>
<tr>
<td>Steel</td>
<td>5900</td>
<td>3230</td>
<td>45</td>
<td>Birks and Green (1991)</td>
</tr>
<tr>
<td>Teflon</td>
<td>1350</td>
<td>550</td>
<td>3</td>
<td>Birks and Green (1991)</td>
</tr>
<tr>
<td>Air</td>
<td>0.4</td>
<td>330</td>
<td>–</td>
<td>4.3 \times 10^{-4}</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>1</td>
<td>1300</td>
<td>–</td>
<td>1.1 \times 10^{-4}</td>
</tr>
<tr>
<td>Oxygen</td>
<td>1</td>
<td>310</td>
<td>–</td>
<td>4.5 \times 10^{-4}</td>
</tr>
<tr>
<td><strong>Food materials</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>1482.3</td>
<td>–</td>
<td>1.48</td>
</tr>
<tr>
<td>Ice</td>
<td></td>
<td>3840</td>
<td>1200</td>
<td>4</td>
</tr>
<tr>
<td>Sodium chloride solution</td>
<td>2.25</td>
<td>1597</td>
<td>–</td>
<td>1.7</td>
</tr>
<tr>
<td>Sucrose solution (10 wt%)</td>
<td>2.25</td>
<td>1511</td>
<td>–</td>
<td>1.57</td>
</tr>
<tr>
<td>Glycerol (10 wt%)</td>
<td>2.25</td>
<td>1527</td>
<td>–</td>
<td>1.63</td>
</tr>
<tr>
<td>Ketchup</td>
<td>2.25</td>
<td>1650</td>
<td>–</td>
<td>1.88</td>
</tr>
<tr>
<td>Apple juice</td>
<td>2.25</td>
<td>1530</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>
TABLE I (continued)
ULTRASONIC PROPERTIES OF SELECTED MATERIALS. UNLESS NOTED, MEASUREMENTS WERE CONDUCTED AT 20°C.

<table>
<thead>
<tr>
<th>Material</th>
<th>Frequency (MHz)</th>
<th>Ultrasonic velocity (m s(^{-1}))</th>
<th>Ultrasonic impedance (10(^6) kg m(^2) s(^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syrup (light and dark corn syrup and maple syrup)</td>
<td>2.25</td>
<td>1854–1900</td>
<td>1.9–2.3</td>
<td>Zacharias and Parnell (1972)</td>
</tr>
<tr>
<td>Olive oil</td>
<td>1.2</td>
<td>1465</td>
<td>1.35</td>
<td>McClements and Povey (1988)</td>
</tr>
<tr>
<td>Corn oil</td>
<td>1.2</td>
<td>1469</td>
<td>1.35</td>
<td>McClements and Povey (1988)</td>
</tr>
<tr>
<td>Palm oil</td>
<td>1.2</td>
<td>1459</td>
<td>1.35</td>
<td>McClements and Povey (1988)</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>1.2</td>
<td>1470</td>
<td>1.35</td>
<td>McClements and Povey (1988)</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>1.2</td>
<td>1471</td>
<td>1.35</td>
<td>McClements and Povey (1988)</td>
</tr>
<tr>
<td>Solid animal fat (31°C)</td>
<td></td>
<td>2000–2070</td>
<td>2.0–2.1</td>
<td>McClements (1997)</td>
</tr>
<tr>
<td>Apple (golden delicious)</td>
<td>0.037</td>
<td>114</td>
<td>0.09</td>
<td>Liljedahl and Abbott (1994)</td>
</tr>
<tr>
<td>Avocado</td>
<td>0.05</td>
<td>274–283</td>
<td>–</td>
<td>Mizrach et al. (1989)</td>
</tr>
<tr>
<td>Papaya</td>
<td>0.12</td>
<td>160</td>
<td>0.16</td>
<td>Hayes and Chingon (1982)</td>
</tr>
<tr>
<td>Potato</td>
<td>0.5</td>
<td>700–850</td>
<td>0.7–0.85</td>
<td>Povey (1989)</td>
</tr>
<tr>
<td>Carrot</td>
<td>0.37</td>
<td>440</td>
<td>0.44</td>
<td>Nielsen and Martens (1997)</td>
</tr>
<tr>
<td>Unyeasted bread dough</td>
<td>0.5</td>
<td>114</td>
<td>141</td>
<td>Povey (1998b)</td>
</tr>
<tr>
<td>Milk chocolate (25°C)</td>
<td>0.5</td>
<td>1020–1740</td>
<td>–</td>
<td>Povey (1998b)</td>
</tr>
<tr>
<td>Aerated milk chocolate (25°C)</td>
<td>0.5</td>
<td>900–1000</td>
<td>–</td>
<td>Povey (1998b)</td>
</tr>
<tr>
<td>De-aerated milk chocolate (25°C)</td>
<td>0.8</td>
<td>2050</td>
<td>–</td>
<td>Povey (1998b)</td>
</tr>
<tr>
<td>Egg thin white</td>
<td>2.5</td>
<td>1560</td>
<td>1.6</td>
<td>Povey (1998b)</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>2.1</td>
<td>1500</td>
<td>1.4</td>
<td>Javanaud (1988)</td>
</tr>
<tr>
<td>Muscle (39°C)</td>
<td>1</td>
<td>1590</td>
<td>1.65–1.74</td>
<td>Goss et al. (1978)</td>
</tr>
<tr>
<td>Cod fillet (30°C)</td>
<td>3.5</td>
<td>1572</td>
<td>55</td>
<td>Ghaedian et al. (1997)</td>
</tr>
<tr>
<td>Skim milk (28°C)</td>
<td>1</td>
<td>1522</td>
<td>1.5</td>
<td>McClements (1997)</td>
</tr>
<tr>
<td>Light cheddar cheese</td>
<td>1</td>
<td>1623</td>
<td>–</td>
<td>Saggin and Coupland (2001c)</td>
</tr>
</tbody>
</table>
gases present, \( K \) has a value between 1.3 and 1.7. Ultrasonic velocity in an ideal gas can also be calculated in terms of its molecular weight, \( M \):

\[
c^2 = \frac{\gamma RT}{M}
\]  

(6)

where \( R \) is the gas constant and \( T \) absolute temperature.

Liquids can readily support longitudinal waves but can only marginally support shear waves. In liquids the difference between adiabatic and isothermal elastic modulus is negligible and the modulus in Eqn (5) is equivalent to the bulk compressional modulus, \( K \). Eqn (5) is often rewritten in terms of the compressibility \( \kappa (= K^{-1}) \), neglecting the imaginary (loss) part:

\[
c^{-2} = \kappa \rho
\]  

(7)

In ideal cases the speed of sound in a mixture is given by the volume fraction weighted sum of the components in Eqn (7). When the beam of sound is narrower than the material through which it passes, there is some shearing at the beam edges, and therefore the modulus is equal to \( K + (4/3)G \). However, since in fluids \( K >> G \), it is often possible to neglect the shear modulus and measurements of longitudinal ultrasonic velocity have little direct value as a measurement of shear rheology.

The elements in a solid are more directly connected than in a fluid; therefore, solids are better capable of supporting both longitudinal and shear waves. When the sound wave passes through a narrow structure where the cross-sectional dimensions are small compared to the wavelength, the speed of longitudinal ultrasound is given by:

\[
c^2 = \frac{Y}{\rho}
\]  

(8)

where \( Y \) is the Young modulus. However, when the longitudinal waves pass through a material in bulk form, some shearing motion occurs at the beam edges and the material shear modulus also affects the wave propagation and, as for liquids, the modulus is given by \( K + (4/3)G \). For shear waves, the speed of sound in solids is equal to \((G/\rho)^{1/2}\) and is typically much slower than for longitudinal waves.

2. Ultrasonic attenuation

Any factors that cause sound energy to be either converted to other energy forms (usually heat) or directed into another direction so they are not
detected will contribute to the attenuation. In practice the measured attenuation can be taken as an arithmetic sum of several contributing factors:

\[ \alpha_{\text{measured}} = \alpha_{\text{classical}} + \alpha_{\text{resonance}} + \alpha_{\text{scattering}} + \alpha_{\text{other}} + \ldots \]  

(9)

where the subscripts refer to the various mechanisms. Measuring attenuation as a function of frequency can help to differentiate between the loss mechanisms and to probe the structure and dynamics of the sample.

The heating and cooling due to the passing pressure wave is not completely efficient. Some energy is lost as heat (molecular friction), which contributes to measured attenuation. Classical attenuation accounts for only viscous and thermal losses:

\[ \frac{\alpha_{\text{classical}}}{\nu^2} = \frac{4\pi^2}{3\rho c} \left( \frac{4\eta}{3} + \frac{(1 - \gamma)\tau}{C_p} \right) \]  

(10)

where \( \eta \) is the viscosity and \( \tau \) is the thermal conductivity. The first term of the equation describes the viscous losses due to intermolecular friction, while the second describes the thermal losses due to inefficient heat transfer. Except for the simplest fluids, other attenuation mechanisms will be much more significant than these, and Eqn (10) usually greatly underestimates the actual attenuation. Important other loss mechanisms include scattering and the disruption of certain chemical equilibria.

When a system is in equilibrium and the conditions change, the position of the equilibrium will also change. A passing ultrasonic wave creates periodic zones of high (and low) pressure and temperature that will tend to cause such a shift in the position of the equilibrium if it can respond rapidly enough. The rate of response of the system will depend on the kinetics of the processes, e.g. for a system at equilibrium between two states interconvertable by first-order processes with rate constants \( k_1 \) and \( k_{-1} \), the relaxation time, \( t_r \) (i.e. the time to respond to a change) is \((k_1 + k_{-1})^{-1}\) (Dickinson and McClements, 1995). More complex expressions exist for more complex transitions. If the rate of change in temperature/pressure is high (high frequency) then the system has no time to respond and there is little additional energy lost, while at low frequency the rate of change in pressure/temperature is slow and the reaction can respond fast enough to remain in equilibrium throughout the passage of the wave. However, at intermediate frequencies the position of the equilibrium is constantly attempting to respond to the varying conditions. At the low and high frequency limits, the ultrasonic velocity is constant (and different) and the attenuation (due to this mechanism) is low. Over the intermediate region the velocity increases from the low to the high frequency limit and there
is a peak in the attenuation spectrum. This process is known as a relaxation. It is expressed mathematically as follows and is illustrated in Figure 2:

\[ c = c_0 + \frac{(c_\infty - c_0) \nu^2}{\nu_r^2 + \nu^2} \] \hspace{1cm} (11)

\[ \alpha_r = \frac{K_r \nu_r^2 \nu^2}{\nu_r^2 + \nu^2} \] \hspace{1cm} (12)

In Eqns (11) and (12) \( c_0 \) and \( c_\infty \) are the ultrasonic velocities at zero and infinite frequency (the plateau values above and below the relaxation), \( \nu_r \) is the frequency of the resonance, \( \alpha_r \) is the attenuation due to the resonance and \( K_r \) is a constant describing its magnitude (Dickinson and McClements, 1995). Measuring the resonance frequency by ultrasonic spectroscopy can allow calculation of the relaxation times of the process and is extremely useful in probing the kinetics of fast chemical reactions (\( t_r \sim 1 \) ns to 1 ms) (Slutsky, 1981; Bryant and McClements, 1999b).
C. ULTRASONIC PROPAGATION IN INHOMOGENEOUS MEDIA

All foods are heterogeneous over some length scales. Very small heterogeneities are not distinguishable by a sound wave and very large structures are best thought of as bulk phases (see sections describing reflection and imaging). Intermediate sized structures where a characteristic length scale is of similar magnitude to the wavelength of the sound can scatter the acoustic wave, leading to increased attenuation and changes in ultrasonic velocity. For longitudinal sound propagating in water, the wavelength of 1 MHz sound is about 1.5 mm, so many very small structures found in food can act as scatterers. The extent of scattering depends on the size, shape, composition and number of inhomogeneities, and so ultrasonic measurements can be used to characterize fine structure.

Mathematical descriptions of scattering interactions are often extremely complex and in many cases of only moderate complexity cannot be readily solved. One successful application of scattering theory is the ultrasonic characterization of emulsions. (Other, more complex, theories are available for nonspherical particles (Ahuja and Hendee, 1978).) Emulsion droplets are spherical and (usually) considerably smaller than the wavelength of sound. These two conditions make the problem much more approachable. The two stages to predicting the ultrasonic properties of a dispersion are: (1) calculate the scattering from an individual particle; and (2) calculate the interactions between the scattered and nonscattered waves from an ensemble of particles.

Because the particle is small compared to the wavelength of the sound, it experiences an effectively uniformly changing pressure. The pressure gradient induces fluid flow and both the particle and the surrounding medium will tend to move. However, because they have a different inertia, the droplet and continuous phase will move slightly out of phase and the particles will be seen to oscillate in the acoustic wave (Figure 3). The moving particle itself acts as a dipolar source, scattering sound mainly forwards and backwards. As well as moving in the pressure gradient, the particle and surrounding media will also be compressed. If the particle has a different compressibility to the surrounding fluid it will be differentially compressed and seen to pulsate. A pulsating droplet acts as a monopolar sound source scattering sound waves homogeneously in all directions (Figure 3).

In fact the actual amount of sound energy scattered by these mechanisms in the long wavelength regime is very small and the losses are dominated by the inefficiencies of the interactions. (Scattering becomes dominant as the particle radius approaches the ultrasonic wavelength.) The friction between the oscillating particle and the surrounding fluid converts sound
energy to heat (viscoinertial losses) and the compression of the particles causes a differential heating effect. Thermal diffusion is inefficient and energy is lost as heat.

Once the scattering from a single particle is understood, the next stage of the problem is to combine their effects to predict the ultrasonic properties of a reasonably concentrated ensemble of particles. The main approaches to this problem are the core–shell models and the use of multiple scattering theories. Both of these are well described in the literature (McClements, 1992; Hermar et al., 1997) and only the former will be described further here.

Multiple scattering theory (Waterman and Truel, 1961) uses an arithmetic sum of terms to calculate the interactions between the primary acoustic wave and the waves scattered from other particles. Each of the terms can be calculated from a series of complex simultaneous equations (McClements and Povey, 1989) and complete solution of the problem can require the extremely laborious calculation of more than 20 of these. However, in the long wavelength limit for moderate concentrations of scatterers, adequate

FIG. 3 Diagram illustrating viscoinertial and thermal scattering from an emulsion droplet in an ultrasonic wave. Viscoinertial scattering is due to the particle oscillating in the continuous phase as a result of the inertial mismatch and acting as a dipolar source. Thermal scattering is due to pulsations of the particle and scatters waves in all directions (i.e. monopolar).
precision can be achieved by considering only the first- and second-order terms (McClements, 1996):

$$\left( \frac{K}{k_1} \right)^2 = 1 - \frac{i4\pi N(A_0 + 3A_1)}{k_1^3} - \frac{48\pi^2 A_0 A_1}{k_1^6}$$  \hspace{1cm} (13)

where $k_1$ is the complex wavenumber of the continuous phase, $N$ is the number of droplets per unit volume ($= 3\phi/4\pi r^3$, $\phi$ is the volume fraction and $r$ the droplet radius). The velocity and attenuation predicted from the theory are contained in the real and imaginary part of $K$, the complex wavenumber of the emulsion ($= \omega/c + i\alpha$). The terms $A_0$ and $A_1$ are analytical solutions to the scattering equations describing the thermal and visco-inertial losses. They are functions of the particle radius, wavenumber (i.e. velocity and attenuation), the physical properties (density, thermal conductivity, viscosity, cubic expansion coefficient and specific heat at constant pressure) of the component phases and the frequency of the sound (McClements, 1996). Using published values for the physical constants it is possible to use Eqn (13) to predict velocity and attenuation as a function of frequency and the concentration and size of the particles present. If necessary, the terms in radius can be replaced by a size distribution.
function – most commonly a log-normal function that only requires the introduction of polydispersity as an additional unknown. Theoretical predictions of the acoustic spectra for various concentrations of fine corn oil droplets in water are shown in Figure 4. (Note that in Figure 4 frequency and particle size are both contained in the x-axis and losses are expressed as attenuation per wavelength, $\alpha \lambda$.) At high and low frequencies the ultrasonic properties of a dispersion are independent of the size of the particles, but over a critical range velocity increases sigmoidally and there is a peak in attenuation. For micrometer-sized droplets this occurs in the range of $10^4$–$10^8$ MHz and size measurements are possible from ultrasonic spectra made in this range. (More complete theories greatly extend this range.)

Particle sizing can then be achieved by measuring the ultrasonic properties of the emulsion over this region and iteratively finding the best value of radius (and perhaps also concentration and polydispersity) to fit the theory to the experimental data. Both velocity and attenuation can be

![Graph showing theoretical prediction of ultrasonic attenuation and velocity spectra for corn oil droplets in water at different concentrations.](image-url)
used for particle sizing (Coupland and McClements, 2001b), although the latter is usually preferred as it is often less sensitive to temperature fluctuations (Chanamai et al., 1998a). These calculations can be somewhat laborious with current computing power and simpler solutions are available under more limited circumstances (Wang and Povey, 1999).

As with any curve-fitting operation, the fewer adjustable parameters (i.e. size, volume fraction, polydispersity and physical constants), the more rapid and satisfactory the solution. Where possible the physical properties of the component phases should be measured independently for the system under investigation, but, failing that, literature values are available (Coupland and McClements, 1997). Babick et al. (2000) used numerical analyses to show the effects of uncertainty in material parameters on the results of a sizing operation. If possible, it is advantageous to determine the volume fraction of the emulsion from an independent chemical or density measurement, leaving only the hard-to-measure microstructural parameters unknown.

The simplification of multiple scattering theory described here has provided a reasonable description of experimental results up to moderate particle concentrations (McClements, 1992), but the full theory is required for concentrated (>~10%) particles or outside the long wavelength limit. More complex theory based around multiple scattering or core–shell theories have been shown to work over a wide range of particle sizes and concentrations (Chanamai et al., 1999; Herrmann and McClements, 1999).

Recently, a theory has been developed to describe the ultrasonic properties of flocculated emulsions (McClements et al., 1998). As we have seen, the compression of particles in a sonic field generates waves of heat that are inefficiently dispersed into the continuous phase. When the droplets are closely associated, for example in a floc, the thermal waves overlap and heat loss is reduced, leading to a decreased ultrasonic attenuation. This effect is treated theoretically by assuming the flocculated emulsion can be treated as a two-phase system, which consists of spherical “particles” (the flocs) dispersed in a continuous phase. The flocs are treated as an “effective medium” whose physical properties (e.g. density, viscosity) depend on the size, concentration and packing of the droplets within them. The ultrasonic properties of the flocculated emulsion can then be calculated by considering the interactions of sound with (a) a single particle within the effective medium of the floc and (b) the floc with the continuous phase. Sample calculations for flocculated emulsions are presented in Figure 5.

In this figure the total oil–water ratio is held as a constant but the proportion of the (1 µm) droplets present in (10 µm) flocs is changed. This theory has been shown to give good agreement with experimental measurements of flocculated oil-in-water emulsions (Chanamai et al., 1998b). At high frequencies the flocs scatter sound themselves and cause
the additional attenuation observed. At low frequencies the thermal overlap effects discussed above are responsible for the reduced attenuation.

D. WAVES AT INTERFACES

Inevitably to make an ultrasonic measurement the wave must pass across an interface where it will be partly reflected (Figure 6). The proportion of transmission and reflectance at an interface is governed by the acoustic impedances of the component phases. The specific acoustic impedance, $Z_a$, is defined as:

$$Z_a = \frac{P}{\xi} = \frac{\omega \rho}{K} = R_a + iX_a$$  \hspace{1cm} (14)

where $P$ is the acoustic pressure. (Note the shear impedance will be different to the longitudinal impedance.) As with all acoustic parameters characterizing both the elastic and inelastic parts of the wave, acoustic impedance...
is complex, expressed as the complex sum of $R_a$ the resistive (real) and $X_a$ the reactive (imaginary) part. In many cases, the attenuation is low and the reactive part of the impedance can be neglected, so it is possible to simplify Eqn (14) to $Z = \rho c$, where $Z$ is the characteristic impedance. The characteristic impedances of some materials are provided in Table I.

The amount of energy reflected at a plane interface can be expressed in terms of the ratios of either the amplitude or the intensity of the reflected (subscript $r$) to the incident (subscript $i$) wave. (In fact it is possible to define the reflection in terms of almost any characteristic parameter, for example the local pressure maximum, particle velocity, particle acceleration.) As intensity ($I$) is proportional to the square of amplitude ($a$), this leads to two commonly used, and frequently confused, reflection coefficients ($R$):

$$R_a = \frac{a_r}{a_i} = \frac{Z_2 - Z_1}{Z_1 + Z_2}$$  \hspace{1cm} (15)

$$R_I = \frac{I_r}{I_i} = \left(\frac{Z_2 - Z_1}{Z_2 + Z_1}\right)^2$$  \hspace{1cm} (16)

When sound that is traveling from a medium of low acoustic impedance encounters a boundary with a second medium of high acoustic impedance (e.g. traveling from air to a solid), the ultrasonic waves are almost entirely reflected ($R$ tends to unity). When the boundary is between media of similar impedance, then $R$ tends to zero and the materials are said to be acoustically matched, or ideally coupled.

By a similar analysis, the transmission coefficient (the ratio of the transmitted and incident waves) is:

$$T = \frac{I_t}{I_i} = \frac{4Z_1Z_2}{(Z_1 + Z_2)^2}$$  \hspace{1cm} (17)

The situation is more complex when the wave encounters an oblique interface and part of the sound is reflected and part of it is refracted, as illustrated in Figure 6(a). The (ultrasonic) refractive index ($n$) is given by Huygens’ principle and Snell’s law as:

$$n = \frac{\sin \theta_1}{\sin \theta_2} = \frac{c_1}{c_2}$$  \hspace{1cm} (18)

where $c_1$ and $c_2$ are, respectively, the velocity of waves in medium 1 and 2, $\theta_1$ is both the angles of incidence and the angle of reflection, and $\theta_2$ is
the angle of refraction. The reflection and the transmittance coefficients are given by:

\[ R = \left( \frac{Z_2 \cos \theta_1 - Z_1 \cos \theta_2}{Z_2 \cos \theta_1 - Z_1 \cos \theta_2} \right)^2 \]  \hspace{1cm} (19)

\[ T = \frac{4Z_1 Z_2 \cos \theta_1 \theta_2}{(Z_2 \cos \theta_1 + Z_1 \cos \theta_2)^2} \]  \hspace{1cm} (20)

When \( \theta_1 \) and \( \theta_2 \) are both equal to 90°, i.e. normal incidence, \( \cos \theta = 1 \) and Eqns (19) and (20) reduce to Eqns (16) and (17).

When longitudinal waves reflect with a certain critical angles at a boundary, they are subjected to a mode conversion and they can generate different types of wave (Rose, 1999). For example, when the velocity of
sound in medium 2 is greater than in medium 1, the refracted longitudinal waves are deviated away from the normal. The first critical angle ($\theta_{cr1}$, Figure 6(b)) occurs when the angle of normal wave refraction $\theta_2$, is equal to $90^\circ$ so that the transmitted longitudinal sound moves along the interface and the shear component continues into the second medium. The second critical angle ($\theta_{cr2}$, Figure 6(c)) is when the shear component is refracted along the interface and the longitudinal component is completely reflected.

E. DIFFRACTION

Our discussion of sound propagation so far has treated the acoustic waves as a coherent beam, and it is possible to find analogous expressions for many of the equations used here in the field of laser optics. However, sound waves are not coherent and we must also account for diffraction. Diffraction is particularly important as beam spreading can mean the detector can receive only a portion of the sound energy produced and the apparent attenuation of the material is overestimated. An ultrasonic transducer can be considered as a piston source. A crystal vibrates under an applied voltage and the force wave propagates away from it as ultrasound. The area in front of the acoustic source is divided in two
regions (Figure 7): the near and far fields. In the near zone, the beam of sound is parallel to the generating source, while in the far zone it diverges apparently from a point at the center of the source. The value of $d_{nz}$, i.e. the limit of the near zone, is calculated from the following equation:

$$d_{nz} = \frac{D^2}{4\lambda}$$

where $D$ is the circular source diameter. Measurements in the far-field region must account for the loss in signal either mathematically (Khimunin, 1972) or through appropriate calibration. Beam spreading can also lead to ultrasonic reflections from the container walls that can make accurate measurement more difficult, particularly as certain angles of incidence lead to mode conversion. In the near-field region the pressure fluctuates wildly with distance and it is extremely difficult to make an accurate measurement (Povey, 1997); it is almost always preferable to make measurements in the far field.

III. MEASUREMENT METHODS

In the theory section above we discussed the various ways that the ultrasonic and material properties of materials are linked. Later we will proceed to describe various applications of these relationships to food
characterization, but we must first consider the practicalities of making ultrasonic measurements. In all cases we seek to define the principles and practicalities of the method as well as provide some suggestions for avoiding common errors. Several important elements of measurement system design are reviewed by McClements and Fairley (1991, 1992), Papadakis (1990a, 1990b), Crecraft (1983), and Sarvazyan (1982). In general, all the measurement systems share some common features:

(a) **Ultrasonic transducers.** Ultrasonic transducers convert an electrical to an ultrasonic signal and vice versa. There are various approaches to transducer design but most depend on the electrically induced vibration of a crystal, i.e. the piezoelectric effect. Cutting the crystal at different angles to the atomic structure can yield transducers with different modes of vibration. When an electrically insulating crystal is compressed it will experience a deformation and electric charges will be created on the surface, i.e. positive charge on one surface and negative on the other, generating an electrostatic field within the crystal. On the other hand, when a crystal is placed in an electric field, it experiences a deformation, i.e. transducers work both as transmitter and receiver. The back of the crystal is usually protected by an acoustically insulating backing material to absorb the energy released in that direction and the front by an acoustically conducting wear plate.

(b) **Signal generator.** A signal generator provides a controlled voltage to the transducer to generate the acoustic signal. The simplest measurements use a board-band electrical pulse to excite the transducer, i.e. an energy pulse containing a wide range of frequencies. This is analogous to striking a bell with a hammer; it is possible to make a loud noise but many frequencies of sound are generated simultaneously. More sophisticated signal generators can provide a burst of known energy composition and/or duration. Acoustic signal generators are frequently quite high voltage (~100 V) and provide a synchronization pulse to zero the time measurement apparatus.

(c) **Digitizer.** The acoustic signal captured and converted to an electrical signal by the transducer must be stored and analyzed. This is commonly achieved using a digital storage oscilloscope or an equivalent computer card. Whatever mode of data capture is selected, it is important to ensure that the resolution is sufficient to capture the high frequency detail in the signal – usually at least three times faster than the highest frequency of interest. The digitizer must also be able to store sufficient data points to capture all the signals of interest and should have a wide dynamic range to cope with highly attenuating samples. Modern oscilloscopes provide many other useful features such as
Fourier domain editing and arithmetic functions. A single ultrasonic measurement is often rapid (~20 µs) so a useful amount of signal averaging can be conducted in apparently “real-time” within the oscilloscope.

All of these items need to be connected with appropriate cabling. Unless very high frequency measurements are considered (>10 MHz), conventional coaxial cabling is sufficient. The cabling should be kept as short as practically possible and should not be changed between calibration and when performing measurements. Defects in connections and cabling can cause errors in measurement often overlooked when troubleshooting a system.

A. PULSED MODES

A real-world application of a pulsed sonic measurement is shouting in a canyon and waiting for the echo to return. Using the wait time (t) and the speed of sound in air (c ~ 330 m s\(^{-1}\)), it is trivial to calculate the width of the canyon (d/2) from:

\[
d = ct
\] (22)

Ultrasonic measurement methods seek to apply this principle in a systemized and controlled manner (McClements and Fairley, 1991). The measurement can be conducted in either one- or two-transducer mode, as illustrated in Figure 8. In one-transducer mode the signal reflects from a material interface and is detected by the original (speaker) transducer; in two-transducer mode the second transducer acts as a receiver. In both

![Diagram illustrating pulsed mode measurements.](image)

FIG. 8 Diagram illustrating pulsed mode measurements. Ultrasonic velocity is calculated from the time taken for the pulse to travel a known distance and attenuation is calculated from the energy loss. Measurements may either be made (a) through transmission mode, where a second transducer is required to detect the signal, or (b) in pulse-echo mode, where a single transducer is used to generate and detect the sound.
cases the ultrasonic velocity is calculated using Eqn (22) as \( c = \frac{d}{t} \). The attenuation of the material can also be measured from the loss in signal amplitude with distance (Eqn (3)), but it is important to account for the nonattenuation losses due to diffraction and imperfect reflection. Frequency dependence of the acoustic parameters can be determined from a Fourier transformation of the various echoes (McClements and Fairley, 1992) or by a series of a few cycles of the pure frequency of interest (tone burst).

The time of flight of the pulse can be measured extremely precisely and so for precise velocity measurements a good measurement of pathlength is also required (see Eqn (23)). Conventional micrometers are not adequately precise so instead the sample cell is calibrated using a material of known ultrasonic velocity (i.e. water; DelGrosso and Mader, 1972). Calibration should be performed regularly and certainly whenever the temperature is changed or the transducers moved. For the most precise measurements it is good practice to check the calibration with another fluid of well-defined properties.

**B. NONCONTACT MEASUREMENTS**

A significant limitation of conventional ultrasonic measurements is the huge impedance mismatch between air and the transducer delay line. Indeed, until recently it was considered impossible to propagate high frequency (~MHz) ultrasound through air. However, a new transducer technology has been developed to overcome the air–transducer impedance barrier (Bhardwaj, 1986). In particular, so-called “quarter-wave matching layers” have been devised which gradually, i.e. layer-by-layer, adapt the traveling waves to lower and lower acoustic impedances until they match that of air and thus provide transmission into air with reasonable efficiency. This procedure has been so successful that it is now possible to transmit sound through air at frequencies of several MHz. The key feature in the transducer construction is the use of new materials, specifically the use of polymers filled with microballoons whose number and size can be adjusted to tailor the acoustic impedance. Ideally the acoustic impedance of the matching layer needs to be equal to the square root of the product of the two impedances to be matched. The thickness of the matching layer needs to be equal to one-fourth of the wavelength of the wave propagating through the layer (Tittmann et al., 1998).

Clearly noncontact measurements are applicable to many sensing applications where direct contact with the food surface is impractical. A typical experimental setup is shown in Figure 9. The ultrasonic signal is passed to one of two ultrasonic transducers arranged a fixed distance
apart, in good alignment with each other. The ultrasonic transducer converts the electrical signal to sound that propagates through the air and is detected by the second transducer at a time $t_1$ later. If there is a sample between the transducers, part of the signal is reflected from the front face of the sample and the reflection detected by the first transducer after an elapsed time of $t_2$. The operation is then repeated in reverse, so measuring a transmitted time signal $t_4$ at the first transducer and, if there is a sample present, an echo at time $t_3$ at the second transducer. Measurements of $t_1$ and $t_4$ are recorded in the absence of a sample and $t_1-t_4$ in the presence of a sample. The thickness ($d$) and speed of sound ($c$) of the sample can be calculated as follows (the superscripts $a$ and $s$ refer to the air and sample, respectively):

$$d_s^2 = c^a t_1^a - \frac{c^a}{2} (t_2^a + t_3^a)$$  \hfill (23)

$$c^s = \frac{-2d_s^2}{(-2t_1^a + t_3^a + t_3^a)}$$  \hfill (24)

(Note that in Eqns (23) and (24), $t_1$ could be replaced by $t_4$ without changing the result.) Attenuation can be calculated from similar measurements of signal power.)
Aside from the technological breakthroughs required in its development, noncontact ultrasonic measurements are a variation of the pulsed methods described above. A key advantage is that thickness is determined directly by the acoustic wave and not measured independently. Precise, contact-mode measurements are restricted to fluids confined in defined geometries and solids can only be measured by (imprecisely) measuring the thickness with calipers.

C. RESONANCE METHODS

Although pulsed modes are the most frequently used methods of ultrasonic food characterization, they have an important disadvantage of requiring a long pathlength to allow the signal to decay sufficiently for an attenuation measurement and for the arrival time to change enough to distinguish velocities. Consequently the sample volumes required are rather large and it can become difficult to maintain adequate temperature control for the highest precision measurements. An alternative approach is to exploit the resonance properties of a container holding the sample (Sarvazyan, 1982). A pair of aligned transducers is placed either directly in the fluid or around a container holding the fluid. One transducer generates a continuous pulse of pure varying frequency and the second acts as the detector. (A second, less common, type of resonator uses a fixed frequency and variable pathlength to generate the resonance peaks. Aside from this, the principles of measurement are similar.) The wave will echo backwards and forwards between the two transducers and when the wavepath is a whole number of half-wavelengths it will constructively interfere and appear as a peak in the frequency/amplitude plot, i.e. the condition for resonance is given by:

$$n \frac{\lambda}{2} = l$$

where $n$ is the (integer) number of the resonance peak, $\lambda$ is the wavelength, and $l$ is the pathlength of the resonator. The wavelength is hard to measure independently, but can be calculated as $c\nu^{-1}$. Therefore the change in resonance frequency ($d\nu$) of a given peak can be related to a change in velocity:

$$\frac{dc}{c} = \frac{d\nu}{\nu}$$

Alternatively, the velocity can be calculated from measurements with a calibration liquid:
\[ c = 2l(v_n - v_{n-1}) \]  

(27)

where \( v_n \) and \( v_{n-1} \) are the resonance frequencies of adjacent peaks. This approach is somewhat simplistic since it neglects the effects of diffraction and nonideal reflection, but these factors can also be dealt with by additional calibration, often using sodium chloride solutions.

The losses of a filled resonance cell are often expressed as the \( Q \)-factor of the system (cell plus sample) calculated from the width of a resonance peak (Buckin and Smyth, 1999). The contribution of the cell to the \( Q \)-factor can be calculated from a suitable attenuation calibration (often magnesium or manganese sulfate solutions) to give the loss properties of the fluid. The \( Q \)-factor of the sample is a function of attenuation:

\[ Q_{\text{sample}} = \frac{\pi}{\alpha \lambda} \]  

(28)

The most precise ultrasonic characterization of fluids is by resonance methods; approaching \( 0.3 \times 10^{-4}\% \) for the best systems (Buckin and Smyth, 1999). Ultra-high precision requires optimization of all of the factors in the measurement system, importantly including temperature. Commercial water baths can easily control temperature to 0.01°C, but ultrasonic velocity precision of the type described above requires a two order of magnitude improvement over this. In practice, precision measurements are made using paired cells in differential mode – one cell measures temperature with a calibration fluid while the other makes high precision measurement of the fluid under consideration.

D. REFLECTANCE METHODS

The magnitude of an echo returning from an interface is related to both the ultrasonic properties* of the two phases (Eqn (16)) and the magnitude of the original pulse. If one of these materials is fixed, then the magnitude of the returning echo is solely dependent on the acoustic properties of the second material and on the magnitude of the incident pulse. This can be exploited as a sensing modality, as changes in the magnitude of an echo returning from the interface between the container and its contents will depend only on changes in the contents. Alternatively, a delay line can be coupled to the transducer and the reflection from the interface between the delay line and the sample surface measured.

In practice it is difficult to ensure that the performance of the transducer and signal generator is constant on a day-to-day basis, so it is better to

*Specifically impedance; these techniques are also known as impedance methods.
normalize the reflection amplitude to that of a calibration material such as water. Alternatively it is possible to use a dual delay line made of two materials linked in series so there are echoes from the interface within the delay line and the delay line–sample interface. The two echoes can be used to solve the two unknowns in the measurements system (i.e. magnitude of the generated signal and ultrasonic properties of the unknown sample). When necessary, reflectance ($R$) can be calculated as a complex parameter from the magnitude ($M$) and phase ($\theta$) of the reflected echo from the sample (subscript s) and calibrant (subscript c) obtained from a Fourier transformation (McClements and Fairley, 1992):

$$R_s = R_c \frac{M_s}{M_c} \exp \left[ i(\theta_s - \theta_c) \right]$$ (29)

Reflectance measurements are often suitable for on-line sensing, as they do not require a fixed and invariant pathlength for the sound within the material of interest, which may not be available in some process equipment. However, reflectance is clearly only sensitive to the surface of material close to the container and may not be representative of the bulk. This may be a particular problem for foods in containers prone to surface fouling. Reflectance is the only effective way to characterize the shear ultrasonic properties of fluids, as shear waves cannot penetrate sufficient distances for transmission measurements.

Related to normal reflectance measurements are the so-called “guided wave” sensors where an acoustic wave is partly trapped inside a container or pipe wall and propagates a significant distance (several meters) along by multiple internal reflections. At each reflection a portion of the sound leaks into the surrounding material so the detected signal is sensitive to changes in composition. Guided wave sensors are particularly valuable in detecting fouling in pipes and hard to reach equipment (Rose, 1999).

E. LOVE WAVE SENSORS

Love waves are shear, horizontally polarized waves that propagate in a thin surface layer. There are some losses into the media surrounding the guiding layer and the wave properties are therefore sensitive to material properties. A major advantage of Love wave sensors is they can be manufactured very cheaply and compactly using printed circuit board technology onto silicon wafers of thickness in the order of 500–1000 µm. They can also be wirelessly interrogated and can operate in pulse echo, through transmission, or in resonance modes.
Curtin and coworkers (1998) developed a micromachined wet cell for Love wave measurements particularly suitable for foods and fluids characterization. A micromachined channel shields all but the wave path from the influence of the liquid and a heater controls the local temperature. A similar design, incorporating paired Love wave sensors, was used by Varadan and Gardner (1999) for a variety of sensing applications. In these cases, one of the sensors is shielded from the fluid and is thus only sensitive to temperature (or mechanical stress), while the other is exposed and responds to both temperature and fluid composition. With two such sensors it is possible to measure two unknowns simultaneously. Arrays of Love wave sensors are particularly exciting food sensors as controlling the masking layer can make them sensitive to different components. In the previous example a single mask allowed simultaneous temperature and fluid property measurement. Using selective masks, similar to those currently employed for ion selective electrodes, it would be possible to develop a genuinely multicomponent ultrasonic sensor.

IV. APPLICATIONS

Even good quality measurements of ultrasonic velocity and attenuation have little value in themselves, but only as they relate to properties determining food quality and to parameters useful for process control. Under certain circumstances the relationships used can be based directly on the underlying physics outlined in the theory section, but in most cases they are empirical. Empiricism should, as always, be treated with caution. Any established link is valid only for the sample set considered and apparently small variations outside this set may render the results meaningless (for example, a correlation between ripeness and ultrasonic velocity for one variety of apple may not hold for another variety). With a mechanistic relationship, it is explicit what is being measured and so wherever possible the physical principles should be at least applied qualitatively to justify an apparent correlation. As we introduce some applications, we shall attempt to stress their theoretical basis wherever possible.

A. LEVEL SENSORS

Perhaps the simplest ultrasonic measurement, one exploiting the gross differences between a fluid and a gas along with the useful ability of sound to make measurements through an opaque solid, is level sensing. Various modes of measurement are available:
(a) **Time-of-flight sensors.** An ultrasonic transducer is placed at the base of the tank and the time taken for a pulse passing up through the liquid and reflecting from the surface is used as a measure of depth. This is precise if the surface is flat and smooth enough to clearly detect a returning echo. This approach has been applied to controlling the filling operation for canned beverages using either a transducer mounted below the fluid (Ridgway et al., 1999) or an air-coupled transducer mounted above (Griffin et al., 2001).

(b) **Presence/absence sensors.** A series of pairs of ultrasonic transducers are aligned with each other at various heights in a column of liquid. The depth of the container is given by the highest pair that can transmit a signal (based on the principle that air cannot support ultrasound but the fluid can. The resolution of this method is limited by the spacing of the transducers.

(c) **Internal reflection sensors.** A pulse of sound is passed obliquely into a bar of solid immersed in the liquid. The sound echoes repeatedly within the solid waveguide and is detected either by a second transducer or in reflectance mode. Each echo depends on the material outside the bar at that point, i.e. either liquid or gas. In general more sound energy will be lost into the liquid so total attenuation of the signal can be related to the amount of the bar immersed in the liquid. Placing the bar appropriately inside a tank can provide a measure of depth.

**B. FLUID FLOW**

Another widely used group of methods determine the rate of flow of liquids from longitudinal ultrasonic velocity measurements. Again, there are three groups of methods (Lynnworth, 1989):

(a) **Transit-time mode** (Figure (10a)). Two transducers are clamped outside a pipe at a known distance from each and angled to the direction of flow either in V-mode (the transducers are mounted on the same side of the pipe; illustrated in Figure (10a)), W-mode (the sound traverses the pipe four times) or in Z-mode (the transducers are mounted on opposite sides of the pipe). By alternately using the paired transducers as transmitters and receivers, two times of flight are measured, with and against the direction of flow. The speed of sound measured with the flow (downstream) is greater than that measured upstream as the translational motion of the fluid supporting the wave adds to (and subtracts from) the movement of the ultrasonic wave. (If the fluid were not moving there would be no difference in measured ultrasonic
The liquid velocity \( V \) in the pipe can be calculated from the difference in time of flight \( \Delta t \) of the sound traveling downstream and upstream through the following equation:

\[
V = BL \Delta t
\]

where \( B \) is a constant and \( L \) is the distance between the two transducers. The constant \( B \) is usually either fixed empirically or calculated as a function of the Reynolds number. Commercial transit-time mode devices are available working in the temperature range –40 to 150°C, in pipe of diameter 1–500 cm, and typically perform best at high flow rates.

(b) **Doppler flowmeters** (Figure (10b)) are based on the principle that the frequency of ultrasonic waves reflected from suspended particles or gas bubbles (discontinuities) within the moving medium is shifted in proportion to the velocity of the moving discontinuities. Current technologies require for a 1 MHz transducer approximately 25 ppm

![Diagram of various types of ultrasonic flowmeter.](image)
of entrained particles in the liquids, and these inhomogeneities should be at least 30 µm in diameter to provide an adequate reflected signal. To obtain a good signal and high precision during measurement, some conditions need to be satisfied; specifically the pipe must be full and flowing above a certain minimum velocity. Also the scattering particles must be moving at the same rate as the liquid. In many cases turbulence is also required.

(c) Vortex shedding (Figure (10c)). A bluff body (the vortex shedder) is fixed to the wall of the pipe, forcing the liquid to flow turbulently around it, producing a series of downstream vortices known as the von Kármán vortex street. These vortices have a characteristic frequency that is proportional to the fluid velocity. Using an ultrasonic device either in transmission or in reflection mode, and knowing the geometry of the shedder and the Reynolds number, the velocity of a liquid can be measured with an accuracy of 1%. Vortex shedders are particularly vulnerable to fouling in fluid streams containing a large amount of suspended solids.

C. TEMPERATURE

In most media the speed of sound is a function of temperature (see Figure 11 for examples) and so measurements of ultrasonic velocity can be potentially used as a thermometer. For example, at room temperature the speed of sound increases approximately 3 m s\(^{-1}\) per degree Celsius. A typical pulse echo instrument is capable of measuring velocity to within 0.1 m s\(^{-1}\), implying a precision in temperature of 0.3°C. (Clearly the more precise resonance methods would be at least an order of magnitude better.) However, when Richardson and Povey (1990) used a tone-burst technique to measure the speed of sound of a fluid flowing in a pipe, they were only able to make measurements within confidence limits of ±1°C. The velocity–temperature function used as a calibration for this technique depends on composition and structure so any changes in the food caused by heating would make the temperature measurements unreliable.

Recently Sigfusson and coworkers (2001) measured the speed of sound in slabs of food undergoing unsteady state cooling. They calculated the theoretical speed of sound as a function of temperature in their food using composition-dependent equations developed by McClements and coworkers (Ghaedian et al., 1998; Chanamai and McClements, 1999; Sigfusson et al., 2001) and showed that the measured values were in good agreement with an integral of this function over the thickness of the slab calculated from the modeled thermal gradient. They further showed measured ultrasonic velocity is a product-specific linear function of core temperature and
therefore a good sensor for product chilling. In a somewhat similar study, Haeeggstrom and Luukkala (2000) measured various ultrasonic properties of fried beef burgers during cooling (74–45°C) and found the best prediction of cooling from the attenuation of 300 kHz sound.

An alternative approach to ultrasonic thermometry is to place a rod in contact with the food. The rod expands with temperature and its length can be accurately measured by propagating an ultrasonic pulse along its length and measuring the reflection from the far (free) end. An advantage of this approach is that it does not need to be recalibrated for each food, but its important disadvantage is that it is invasive. Richardson and Povey (1990) evaluated several rod shapes and were able to achieve confidence limits to their measurements of 0.3°C using a tapered design.

D. COMPOSITION

Some of the most simple and most widely used ultrasonic applications are composition sensors. The speed of sound in many solutions increases nonlinearly with increasing concentration; several examples are shown in

FIG. 11 Speed of sound (~1–2 MHz) as a function of temperature for (○) distilled water, (△) 20% sucrose solution, (●) corn oil, and a fine corn oil-in-water emulsion (■) φ = 10%, (□) φ = 50%. Note that while the speed of sound in water and aqueous solutions tends to increase (to a maximum at ~70°C) with temperature, it decreases in oils. The ultrasonic velocity in mixed oil–water systems shows a response to temperature somewhere between the constituents depending on their volume fraction. At the temperature where the speed of sound is similar in oil and water (~20°C), it is insensitive to their ratio in a mixed system. Data were either measured by the authors or taken from various literature sources.
<table>
<thead>
<tr>
<th>Food</th>
<th>Ultrasonic parameters</th>
<th>Measurement conditions</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Several simple sugars</td>
<td>Velocity (by resonance 1 MHz)</td>
<td>20–45°C</td>
<td>Study of hydration properties of sugars</td>
<td>Shiio (1957)</td>
</tr>
<tr>
<td>Alcohol and solids in wine</td>
<td>Velocity</td>
<td>30 and 65°C</td>
<td>Good correlation ($r^2=0.99$) with composition</td>
<td>Winder et al. (1970)</td>
</tr>
<tr>
<td>Yeast slurry and wort (°Plato) in brews</td>
<td>Velocity (3.6 MHz)</td>
<td>20°C</td>
<td>Calibration curve for wort and yeast and then measurements in brews</td>
<td>Feil and Zacharias, (1971)</td>
</tr>
<tr>
<td>Fruit and vegetable juices, oils, sauces, wines, syrups</td>
<td>Velocity</td>
<td>5–50°C</td>
<td>Effect of temperature, water content and alcohol content on ultrasonic velocity</td>
<td>Zacharias and Parnell (1972)</td>
</tr>
<tr>
<td>Sugar in solution and in juices</td>
<td>Velocity (0.1–10 MHz)</td>
<td>10–110°C</td>
<td>Error ±0.25%</td>
<td>Fedotkin et al. (1981)</td>
</tr>
<tr>
<td>Aqueous solutions of starch and gelatin</td>
<td>Absorption</td>
<td>30–60°C</td>
<td>Excess absorption was noted in starch solutions at various concentrations and temperatures</td>
<td>Reddy and Suryanarayana (1981)</td>
</tr>
<tr>
<td>Various monosaccharides</td>
<td>Velocity</td>
<td>20–80°C, 0–1.2 m</td>
<td>Adiabatic compressibility was calculated from velocity and density.</td>
<td>Smith and Winder (1983)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Velocity and absorption (5–25 MHz)</td>
<td>20–40°C, 0–5 mm</td>
<td>Molar compressibility and volume were calculated from velocity and density</td>
<td>Berchiesi et al. (1987)</td>
</tr>
<tr>
<td>Monosaccharides, disaccharides, and methyl pyranosides</td>
<td>Velocity</td>
<td>5, 15, 25°C, 0–50 mm</td>
<td></td>
<td>Kaulgud and Dhondev (1988)</td>
</tr>
<tr>
<td>Food</td>
<td>Ultrasonic parameters</td>
<td>Measurement conditions</td>
<td>Comments</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------------</td>
<td>------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Nucleic bases</td>
<td>Velocity (7.1–7.4 MHz)</td>
<td>15–35°C, 0.5–1.5 mg per g H₂O</td>
<td>Apparent molar adiabatic and isothermal compressibility were calculated for seven nucleic basis</td>
<td>Buckin (1988)</td>
</tr>
<tr>
<td>Sugar content in fruit juices and beverages</td>
<td>Velocity (2.5 MHz)</td>
<td>10–30°C</td>
<td>Ultrasonic measurements predicted sugar content to within 0.2% (pure sugar) or 0.5% (mixed sugars) and was sensitive to sugar type</td>
<td>Contreras et al. (1992)</td>
</tr>
<tr>
<td>Cod fillet</td>
<td>Velocity and attenuation (3.5 MHz)</td>
<td>5–35°C</td>
<td>Relation between moisture and speed of sound ($r^2 &gt; 0.8$). No relation with attenuation</td>
<td>Ghaedian et al. (1997)</td>
</tr>
<tr>
<td>Catfish, cod, flounder, mackerel and salmon</td>
<td>Velocity and attenuation (3.5 MHz)</td>
<td>5–35°C</td>
<td>Measurement at different temperatures for multicomponent (fat, moisture, ash) analysis</td>
<td>Suvanich et al. (1998)</td>
</tr>
<tr>
<td>Fish analogs made from dried cod powder, sunflower oil and water</td>
<td>Velocity and attenuation (3.5 MHz)</td>
<td>5–35°C</td>
<td>Measurement at different temperatures for multicomponent (fat, moisture, ash) analysis</td>
<td>Ghaedian et al. (1998)</td>
</tr>
<tr>
<td>Atlantic mackerel</td>
<td>Velocity and attenuation (1–6 MHz)</td>
<td>5–25°C</td>
<td>Measurement at different temperatures for multicomponent (fat, moisture, ash) analysis. Attenuation was insensitive to food composition</td>
<td>Sigfusson et al. (2001)</td>
</tr>
</tbody>
</table>
### TABLE II *(continued)*  
REPORTED DETERMINATIONS OF COMPOSITION FROM ULTRASONIC MEASUREMENTS

<table>
<thead>
<tr>
<th>Food</th>
<th>Ultrasonic parameters</th>
<th>Measurement conditions</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sausages</td>
<td>Velocity (1 MHz)</td>
<td>4–25°C</td>
<td>Explained variance 99.6% for fat, 98.7% for moisture and 85.4% for protein.</td>
<td>Benedito et al. (2001)</td>
</tr>
<tr>
<td>Glycerol, sucrose, sodium chloride, tomato</td>
<td>Velocity and reflectance (2.25 MHz)</td>
<td>20°C</td>
<td>Both velocity and reflection coefficient could be used as concentration sensors with comparable precision.</td>
<td>Saggin and Coupland (2001b)</td>
</tr>
</tbody>
</table>
Figure 12(a) and Table II. Clearly any of these correlations could be used, with caution, as a calibration curve for a concentration. The first major problem is that ultrasonic velocity is a function of overall composition as well as other factors, importantly including microstructure and temperature, and so concentration measurements require the other factors to remain unchanged. It is usually impossible to measure multiple components simultaneously. Ultrasound can, however, be combined as
part of a measurement suite, for example for the simultaneous determination of alcohol and sugars in beverages from combined velocity and density measurements.

One way for making multicomponent measurements using ultrasound exploits the different temperature dependencies of the speed of sound in oil and water (Figure 11). At approximately 20°C, the speed of sound in an oil–water system is independent of the concentration of oil and so measurements at this temperature can be used to measure the composition of the aqueous phase (Chanamai et al., 1998a). Moving away from this temperature the dependence on oil/water ratio increases and can be measured. This principle has been used in a dairy products analyzer developed in the United States by Winder and coworkers (1961) and the BIOTEST instrument developed in the Soviet Union (cited in Buckin and Smyth, 1999). It has also been used for the determination of fat and non-fat solids in various fish (Ghaedian et al., 1997, 1998; Suvanich et al., 1998; Sigfusson et al., 2001) and meat products (Chanamai and McClements, 1999; Benedito et al., 2001) and for the determination of alcohol in wine (Winder et al., 1970).

Other ultrasonic parameters can also be used to measure concentration, but it should be stressed these often covary with ultrasonic velocity so may not be compatible as multicomponent sensors. Recently we have shown (Saggin and Coupland, 2001b) that the proportion of a longitudinal ultrasonic pulse that is reflected from the surface of a variety of foods depends on concentration (Figure 12) and that the precision available using a simple pulsed methodology was comparable to that for velocity-based measurements (for example, the amount of water added to a ketchup sample could be determined to within 2% from velocity measurements and within 1.5% from reflectance). Reflectance measurements can be a better technique for on-line analysis when the geometry of the system is not conducive to velocity measurements, but only in cases where the surface is representative of the bulk. Ultrasonic attenuation tends to increase only slightly with concentration for many food solutions and so is rarely used as a concentration sensor. However in some cases, particularly for some ionic solutions and for dispersions, attenuation has a useful dependency on concentration.

An especially important application of composition sensing is using ultrasonic measurements to predict the composition of animals and carcasses. One of the simplest versions of this is exploiting the correlation between backfat thickness (measurable ultrasonically) and bulk carcass composition (Fisher, 1997). Knowing the different speed of sound for lean and fat tissue, it is also possible to measure fat deposition in the carcass. However, ultrasonic velocity in muscle tissue is a function of both composition and
microstructure (texture), so the results may be somewhat convoluted (Abouelkaram et al., 2000).

E. PHASE TRANSITIONS

The speed of sound is typically much greater in a solid than in a liquid, and thus ultrasonic sensors are particularly applicable to monitoring phase transitions in both lipid- and water-based systems. There are two main groups of methods – isothermal and temperature scanning – although these are based on the same principle.

1. Isothermal.

Various authors (Miles et al., 1985; McClements and Povey, 1987) have shown that the speed of sound in liquid oil increases by approximately 3 m s⁻¹ per 1 wt% increase solid fat present and exploited this to measure fat crystallization (Coupland, 2001). If ultrasonic velocity can be measured to within 0.1 m s⁻¹, then this implies that a change of as little as 0.03% in solids content can be determined ultrasonically. Some theoretical justification for the observed data can be gained by extending Eqn (7) for a two-phase system by using volume fraction weighted averages of density and adiabatic compressibility: \( \kappa = (1 - \phi)\kappa_1 + \phi\kappa_2 \) and \( \rho = (1 - \phi)\rho_1 + \phi\rho_2 \) (subscripts 1 and 2 refer to the dispersed and continuous phases, respectively). The compressibility of each phase can be calculated from independent measurements of velocity and density and the solid fat content (SFC) as the positive solution of the quadratic equation:

\[
SFC = \frac{-B - \sqrt{B^2 - 4AC}}{2A}
\]

where

\[
A = c_1^2 \left(1 - \frac{\rho_1}{\rho_2}\right) + c_2^2 \left(1 - \frac{\rho_2}{\rho_1}\right)
\]

\[
B = c_2^2 \left(\frac{\rho_2}{\rho_1} - 2\right) + c_1^2 \frac{\rho_1}{\rho_2}
\]

\[
C = c_2^2 \left(1 - \frac{c_1^2}{c^2}\right)
\]

and \( c \) is the speed of sound in the semi-solid fat. This model is based on several at least partly unrealistic assumptions (i.e. small density difference between phases and solids fat particles much smaller than ultrasonic
wavelength), yet gave a good prediction up to approximately 20% solids (McClements and Povey, 1987).

These workers also showed that this method of SFC determination was superior to NMR for low levels of solids (McClements and Povey, 1987). However, an important practical difficulty arises at higher solid fat content when the fat begins to scatter sound significantly, perhaps from voids within the structure (Cebula et al., 1990) or from air entrained in the viscous mixture, and it becomes extremely hard to make a measurement. Another problem with many fats (particularly cocoa butter) is that the contraction of solidification is sufficient to pull the sample away from the wall of the container and form an air gap large enough to prevent ultrasonic propagation. Despite these difficulties, Garbolino et al. (2000) used an ultrasonic velocity meter as an on-line sensor to demonstrate the effect of applied shear on the onset of lipid crystallization.

Neither of these disadvantages arises when the oil is present as the dispersed phase of an emulsion, and some of the strongest applications of SFC determination have been made in dispersed systems. The relevant theory is largely similar and McClements (1988) showed that the ultrasonic velocity in a mixed alkane oil emulsion increases almost linearly with SFC. The technique has been used to track the crystallization kinetics of emulsified alkanes (Hindle et al., 2000), palm oil (Hodate et al., 1997; Kloek et al., 2000) and cocoa butter (Hindle et al., 2000). Most of the published work reported in this field suggests that the increase in ultrasonic velocity with solid fat is largely independent of solids composition (i.e., polymorphism), but Kloek and coworkers (2000) have suggested there may be some dependency on the polymorphic form of the fat. As polymorphic forms differ in density and crystal packing, it seems likely that there is at least some effect.

The same principles apply equally well for aqueous phase transitions, but have been applied less widely. Miles and Cutting (1974) showed that the speed of sound is much greater in frozen meat and thus the solid/liquid ratio (and thus enthalpy) of partly frozen product could be calculated from an ultrasonic velocity measurement.

2. Temperature scanning measurements

Changes in ultrasonic properties, usually velocity, measured as a function of temperature, can be used to identify phase transitions in lipid and aqueous systems. For example, the speed of sound in a lipid system is changed by temperature according to two factors: (1) the speed of sound in liquid oils decreases with temperature (Figure 11) and (2) the speed of sound in oils
increases with SFC. These factors overlay to produce a typical sigmoidal function, as shown in Figure 13. The solid fat content can be measured by extrapolating the lines from the solid and liquid regions over the transition range then calculating SFC as:

\[
\text{SFC} = \frac{(1/c^2_s) - (1/c^2_l)}{(1/c^2_s) - (1/c^2_l)}
\]

where \( c \) is the measured ultrasonic velocity and \( c_s \) (\( c_l \)) the velocity in pure solid fat (liquid oil) extrapolated to measurement temperature (Miles et al., 1985). Eqn (32) has a similar theoretical basis as Eqn (31) and relies on similar, partially justified, assumptions. This approach is comparable to differential scanning calorimetry in that it assumes that the extrapolations drawn are from pure liquid or solid fat and suffers from similar limitations.

The temperature scanning approach has also been applied to emulsified oils and has identified transitions in alkanes (McClements et al., 1993; Hindle et al., 2000) and triglycerides (Hindle et al., 2000; Kloek et al., 2000). Although the changes in attenuation associated with dispersed phase transitions tend to be more modest than those seen in velocity measurements, and thus less widely reported, McClements et al (1993) noted an anomalously high attenuation as emulsified alkanes melted. The magnitude of this peak decreased with frequency (0.5–3.5 MHz). These workers
postulated that the ultrasonic energy was absorbed by perturbing the \( \text{fat}_{\text{crystal}} \leftrightarrow \text{oil}_{\text{liquid}} \) equilibria and was thus related to the molecular dynamics of melting.

Other workers have used temperature scans of other ultrasonic properties to measure phase transitions. Recently Garbolino and coworkers (2000) showed that it is possible to measure the onset of lipid crystallization by the changing reflection coefficient of longitudinal ultrasonic waves. Shore and others (1986) that showed the attenuation coefficient was approximately four times larger in frozen beef than in unfrozen samples, but no attempt was made to measure percentage frozen from the data. Although not classically a phase transition, Mulet et al. (1999) showed the temperature dependency of the ultrasonic velocity in cheese changes as the cheese melts and were able to identify three distinct zones that could be related to thermal events in a thermogram.

**F. RHEOLOGICAL PROPERTIES**

There have been many efforts made to correlate the texture of foods, particularly fruits and vegetables, with ultrasonic properties; some of these investigations are summarized in Table III. The correlation coefficients reported are often quite low and probably fortuitous. The factors that allow sound to propagate through complex biological structures are poorly understood and there is no reason to assume they will correlate with the factors that determine bulk texture. This problem is compounded in most fruit and vegetables by the strong scattering of sound by intracellular air, which makes transmissions measurements at high frequencies extremely difficult. Lower frequency sound tends to be attenuated less and several of the publications listed in Table III use sonic resonance techniques, a related method based on measurements of the frequencies generated by the fruit when struck.

The limitations of some of these approaches arise from the lack of mechanistic basis for a relationship for the relationships sought. Longitudinal waves in fluids are only weakly related to changes in shear modulus and as \( K \gg G \), longitudinal ultrasonic properties are effectively exclusively dependent on bulk modulus. Bulk modulus is rarely measured for foods as it is thought to have limited practical significance and it is unsurprising that attempts to correlate longitudinal ultrasonic parameters with shear viscous properties have met with only limited success. Another significant problem is that there is no reason to expect the ultrasonic properties of a food to be at all related to the large-deformation or failure properties of a food (e.g. fracture, chewability) and again the correlations seen are indirect, although frequently very useful. An example of this type of relation was
TABLE III
REPORTED DETERMINATIONS OF FOOD TEXTURE FROM SONIC MEASUREMENTS.

<table>
<thead>
<tr>
<th>Food</th>
<th>Ultrasonic measurement</th>
<th>Texture property</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apples</td>
<td>Bar velocity, $V_b$; apparent wave propagation, $V_{ap}$; dilatation velocity, $V_d$</td>
<td>Poisson ratio ($\nu$), density, Young modulus ($E$), shear modulus ($G$)</td>
<td>The velocity of propagation through the whole fruit is related to velocity through quarter sections</td>
<td>Garrett and Furry (1972)</td>
</tr>
<tr>
<td>Apples</td>
<td>(a) Hitting sound wave and (b) power spectrum (20–1.25 kHz)</td>
<td>Mechanical firmness and sensory data</td>
<td>Best correlation, $r = 0.77$</td>
<td>Yamamoto et al. (1980)</td>
</tr>
<tr>
<td>Apples (red delicious)</td>
<td>Acoustic transmission profile (by sonic resonance) (5–10 000 Hz)</td>
<td>Firmness, soluble solids, and maturity</td>
<td>Single and multiple frequency models were developed to compare acoustic data with mechanical methods.</td>
<td>Affeldt and Abbott (1989)</td>
</tr>
<tr>
<td>Apples (golden delicious)</td>
<td>Amplitude resonance (0–2 kHz)</td>
<td>Puncture test</td>
<td>Resonance frequencies showed relationship to apple softening. Resonance method was more reproducible than puncture method</td>
<td>Liljedahl and Abbott (1994)</td>
</tr>
<tr>
<td>Watermelon</td>
<td>Amplitude resonance (0–2 kHz)</td>
<td>Puncture test</td>
<td>Best correlation, $r = 0.73$</td>
<td>Liljedahl and Abbott (1994)</td>
</tr>
<tr>
<td>Biscuits</td>
<td>Velocity, attenuation</td>
<td>Mechanical tests and sensory brittleness</td>
<td>Poor correlation with sensory data ($r = 0.13$) and only weak with mechanical data ($r &gt; 0.5$)</td>
<td>Povey and Harden (1981)</td>
</tr>
</tbody>
</table>
TABLE III (continued)
REPORTED DETERMINATIONS OF FOOD TEXTURE FROM SONIC MEASUREMENTS.

<table>
<thead>
<tr>
<th>Food</th>
<th>Ultrasonic measurement</th>
<th>Texture property</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oranges (skin texture)</td>
<td>Reflectance</td>
<td></td>
<td>Reflectance depends on skin roughness</td>
<td>Sarkar and Wolfe (1983)</td>
</tr>
<tr>
<td>Tomatoes (cracks)</td>
<td>Reflectance</td>
<td></td>
<td>Significantly more scattering in reflectance from cracked tomatoes, correlated ( r = 0.71 ) with crack width</td>
<td>Sarkar and Wolfe (1983)</td>
</tr>
<tr>
<td>Potato, avocado, carrot</td>
<td>Velocity (50 kHz), attenuation (50 kHz), reflectance (500 kHz)</td>
<td></td>
<td>Large velocity difference between fresh and ripe avocado</td>
<td>Mizrach et al. (1989)</td>
</tr>
<tr>
<td>Winter-grown melons</td>
<td>Velocity, attenuation (50 kHz)</td>
<td>Modulus of elasticity, and tangent modulus</td>
<td>Poor correlation of velocity with internal properties of melons.</td>
<td>Mizrach et al. (1991)</td>
</tr>
<tr>
<td>Avocado</td>
<td>Velocity, attenuation</td>
<td>Firmness</td>
<td>Distinct minimum in velocity during ripening possibly related to changes in internal structure</td>
<td>Mizrach et al. (1996)</td>
</tr>
<tr>
<td>Avocado</td>
<td>Velocity, attenuation</td>
<td>Firmness</td>
<td>Correlation between firmness and attenuation related to cold-softening of avocado</td>
<td>Flitsanov et al. (2000)</td>
</tr>
</tbody>
</table>
### TABLE III (continued)
REPORTED DETERMINATIONS OF FOOD TEXTURE FROM SONIC MEASUREMENTS.

<table>
<thead>
<tr>
<th>Food</th>
<th>Ultrasonic measurement</th>
<th>Texture property</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avocado</td>
<td>Velocity, attenuation</td>
<td>Dry weight during ripening</td>
<td>Negative correlation between attenuation and dry weight</td>
<td>Mizrach et al. (1999)</td>
</tr>
<tr>
<td>Mango</td>
<td>Velocity, attenuation</td>
<td>Softness, acidity, and sugar content during ripening</td>
<td>Statistical model used to relate changes in ultrasonic signal to ripening in a packing house</td>
<td>Mizrach et al. (1997)</td>
</tr>
<tr>
<td>Cooked carrots</td>
<td>Velocity, attenuation (37 kHz)</td>
<td>Compressive Young modulus and strain at failure</td>
<td>Major changes in all properties in the first 25 min of cooking associated with changes in air and water content.</td>
<td>Nielsen and Martens (1997)</td>
</tr>
<tr>
<td>Mahon cheese</td>
<td>Velocity (1 MHz)</td>
<td>Texture profile analysis</td>
<td>Ultrasonic velocity decreases with curing time probably due to drying. The authors predicted a model (92% of variance) of the dependence of velocity on temperature and deformability modulus. Best measurement temperatures 0–17°C</td>
<td>Benedito et al. (2000c)</td>
</tr>
<tr>
<td>Cheddar cheese</td>
<td>Velocity (1 MHz)</td>
<td>Texture profile analysis</td>
<td></td>
<td>Benedito et al. (2000b)</td>
</tr>
</tbody>
</table>
seen by Benedito et al. (2000a), who showed the speed of sound increased (approximately 1650–1720 m s\(^{-1}\)) in Mahon cheese during aging up to 1 year along with instrumental measures of large-deformation elasticity and yield values. Although the correlations were good (0.92 with modulus and 0.88 with the force maximum) the cheeses were also shown to lose about 30% of their water content over the aging period. These authors recognize that the value of ultrasound as a cheese texture sensor in this case is most likely due to both the speed of sound and texture varying with water content (Benedito et al., 2000c) and showed similar results with Cheddar cheese (Benedito et al., 2000b). A similar correlation-type approach was taken by Povey and Harden (1981) to relate the crispness of cookies to ultrasonic velocity.

An interesting way around both of these problems was taken by Faeth and Chem (1999), who used low frequency (0.1 MHz), noncontact ultrasonics to measure the position of the surface of baked goods as they were deformed by a jet of air. In this case ultrasound was simply being used as a strain gauge and the ultrasonic properties of the food were not measured. These workers were able to correlate the induced deformability of the bread with baking time (\(r = -0.85\)).

A more direct set of texture measurements is based on the direct relationship between shear acoustic properties and fluid viscosity. Viscous liquids are only marginally able to support shear waves and very strongly attenuate them. For low viscosity liquids (~1 cP), the shear velocity and attenuation coefficient are expressed as follows (Blitz, 1963):

\[
c^2 = \frac{2\eta\omega}{\rho} \quad (33)
\]

\[
\alpha = \sqrt{\frac{\omega\rho}{2\eta}} \quad (34)
\]

Both speed of sound and attenuation are frequency dependent and, while speed of sound increases with increasing viscosity, the attenuation decreases. For example, for water according to Eqns (33) and (34), 1 MHz shear waves would have a velocity of 3.5 m s\(^{-1}\) and an attenuation of 15 000 dB mm\(^{-1}\), making it impossible to transmit waves over even a few micrometers. As the viscosity increases the characteristic time for the shear wave to collapse becomes less than the (reciprocal) frequency of the wave and propagation becomes more possible (Blitz, 1963).

One solution to this measurement problem is to generate a pulse of shear waves in a solid and measure the amount of this pulse reflected from
an interface with water. Recently Kulmyrzaev and McClements (2000) and Saggin and Coupland (2001a) took this approach to measuring the viscosity of honey and oils, respectively. Both groups reported good correlations between ultrasonically and conventionally measured data and Kulmyrzaev and McClements noted the magnitudes of the viscosity measured by the two methods were significantly different – perhaps due to a relaxation over the wide span of frequencies separating the two methods.

Buckin and coworkers (Buckin and Kudryashov, 2001; Kudryashov et al., 2001) used a more sensitive shear wave resonator to track the sol–gel transition in milk during yogurt manufacture. The formation of a gel was monitored in time using high-frequency shear wave transducers (5–15 MHz) and dynamic rheology at a frequency of 0.1 Hz. Both methods monitored the same kinetics of formation of a gel network, which was accompanied by a significant increase of the elastic and storage modulus. However, the absolute values of \( G' \) and \( G'' \) of the casein gel at frequencies of 5 and 15 MHz were approximately 1000 times higher than the values at the low frequency (0.1 Hz). Furthermore, the measured viscosity at high frequencies was closer to the viscosity of liquid milk. The interpretation given by these workers was that at high frequencies (time scale \( 10^{-7} \)–\( 10^{-8} \) s) the movements of the gel network are “frozen” and do not contribute to the viscous losses.

Lee and coworkers (1992) were, somewhat surprisingly, able to propagate shear waves over reasonable distances (1.1 cm) in bread dough and cheese. The shear wave measurements were used to calculate \( G' \) and \( G'' \) and there was reasonable qualitative agreement between the acoustic and conventional measures of material texture; however, the quantitative relationship was weak. More recently Letang and coworkers (Letang et al., 2001) combined longitudinal velocity and shear wave resonance to study the effect of water content on complex shear and longitudinal moduli of bread dough and showed that the magnitude of the moduli increase with frequency.

Love waves are also shear ultrasound, and Varadan and Gardner (1999) noted a concentration and viscosity-dependent change in attenuation of 0.5 dB wt%\(^{-1}\) on inserting a micromachined liquid cell into glycerol solutions. A Love wave sensor is a surface measurement and again if the material close to the sensor is not representative of the bulk will not be reliable.

An interesting and novel approach to the ultrasonic characterization of fluid viscosity was recently taken by McCarthy and Choi (personal communication) based on the principle that the velocity profile across a fluid flowing in a pipe is dependent on both the rheological properties of the fluid and the pressure drop across the pipe. Pressure drop is easily measured by nonultrasonic methods and any method capable of measuring a flow profile could be used to calculate the rheological properties of interest.
There are two observations that are used to characterize fully developed, steady laminar flow in tomographic-based viscometry. First, independent of the constitutive equation for the material, the conservation of linear momentum demands that the shear stress, $\sigma$, depends upon the radial position in the tube, $r$:

$$\sigma(r) = -\frac{\Delta P}{2L} r$$  \hspace{1cm} (35)

where $\Delta P/L$ is the pressure drop taken from the downstream direction of the flow over the tube length $L$. Second, the shear rate, $\dot{\gamma}$, is given by:

$$\dot{\gamma}(t) = \left| \frac{d\nu(r)}{dr} \right|$$  \hspace{1cm} (36)

and is locally computed using a global curve fit to the tomographic velocity data. Using both Eqns (35) and (36) at each radial position yields the shear stress as a function of shear rate. Repeating this evaluation at all radial positions in a single image of velocity profile, the fluid viscosity can be characterized over a wide range of shear rates. Theoretically the shear rate ranges from zero at the tube center to a maximum at the tube wall. Then the shear viscosity, $\eta(\dot{\gamma})$, is given by:

$$\eta(\dot{\gamma}) = \frac{\sigma(\dot{\gamma})}{\dot{\gamma}}$$  \hspace{1cm} (37)

A transducer is placed at an angle to the pipe wall and is used to transmit and receive pulsed ultrasound. The transducer used in this system operates at 5 MHz in a pulse mode. The time from transmission to reception yields the distance the pulse has traveled and the frequency shift of the wave yields the velocity of the particle reflecting the wave. The particles are assumed to move at the speed of the fluid and hence the measurement yields the velocity profile of the fluid. Ultrasonic Doppler velocimetry (UDV) measurements of the fluid velocity profile are in excellent agreement with simultaneous magnetic resonance imaging measurements of the velocity profile. Figure 14 demonstrates the UDV velocity image for tomato sauce and the extracted velocity profile.

G. DISPERSED SYSTEMS AND FOOD MICROSTRUCTURE

The ultrasonic properties of dispersed systems depend on the frequency of the sound and the size, shape and number of scattering particles (Figure 4).
FIG. 14 Ultrasonic Doppler velocimetry of the flow pattern of tomato concentrate suspension (4.2° brix) and the calculated velocity profile. Note the decrease in signal intensity across the velocity image due to signal attenuation. Signal attenuation does not adversely affect the estimation of rheological parameters as long as the velocity profile can be extracted. (Figure courtesy of Dr M. McCarthy and Y.C. Choi, University of California, Davis, CA.)
In food emulsions the scattering theory has been developed to such an extent that good predictions can be made of the ultrasonic properties of a wide range of emulsion sizes and concentrations. These devices are particularly interesting as they remain the only practical technology for the rapid determination of the particle size of realistic concentrations of emulsions and are commercially available from a number of sources. Some examples of successful particle sizing include: emulsified vegetable oils (Coupland and McClements, 2001b), salad dressings (McClements et al., 1990; Chanamai et al., 2000), milk fat globules (Miles et al., 1990), and prenatal fat emulsions (Kippax et al., 1999). Similar methods have also been used to measure the size of casein micelles (Povey, 1997; Povey et al., 1999).

A number of experimental studies have shown that droplet flocculation causes an alteration in the ultrasonic properties of emulsions (McClements, 1994; Hermar et al., 1997; Hibberd et al., 1997). This theory has been applied to the study of droplet aggregation in protein-stabilized emulsions in which flocculation was induced by decreasing the electrostatic repulsion between droplets (Demetriades and McClements, 1999) or by adding a nonabsorbing biopolymer to the continuous phase (Chanamai et al., 1998c; Chanamai and McClements, 2001). These studies have shown that ultrasound is sensitive to the spatial distribution of the droplets within an emulsion. The same ultrasonic spectroscopy technique has been used to study the disruption of flocs in a shear field (Chanamai et al., 1998c). As the emulsions are exposed to higher shear rates the flocs become disrupted and their attenuation spectra becomes closer to that of nonflocculated droplets.

An interesting modification of the scattering theories introduced above allows the calculation of the emulsion \( \zeta \)-potential. Briefly, the acoustic wave causes a charged particle to oscillate within its associated ionic environment. At certain frequencies the ions cannot realign rapidly enough to keep pace with the moving particle and the ionic friction generates a voltage and causes additional ultrasonic losses, i.e. the electroacoustic effect (Dukhin et al., 2000). Instrumentation can be based on this principle by either measuring the current generated from the particles in an applied ultrasonic wave or the ultrasound generated by the particles in an oscillating electrical field. The latter method is believed to be the most practical as the ultrasound generated by the particles is directly proportional to their electrophoretic mobility. Electroacoustic techniques have been applied to the determination of \( \zeta \)-potential in concentrated suspensions including oil-in-water emulsions (Kong et al., 2001), casein micelles (Wade et al., 1996) and dairy emulsions (Wade and Beattie, 1997).

Bubbles and concentrated foams are important structures common to many foods. In many cases the foam is an integral and valued feature (e.g.}
bread, ice cream, beer) but in other cases excessive foaming is a problem during processing (e.g. in bioreactors). Foams are transient and fragile and thus very difficult to characterize. Gravimetric analysis can usually provide a good estimate of volume fraction, but size analysis usually requires optical microscopy or other imaging techniques. Ultrasound is extremely sensitive to both the volume fraction and size of air bubbles and is a promising analytical approach. However, the extremely high attenuation means it is practically impossible to transmit high-frequency sound through an appropriate thickness of foam. Reflectance is a promising alternative and Kulmyrazaev et al. (2000) measured the reflectance spectra (1–7 MHz) from a series of model food foams and noted clear spectral differences between the foams (different sizes and concentrations), but only qualitative agreement with theoretical calculations.

Foreign bodies, such as glass, steel, plastic, wood or stones, can be hard to detect in the finished product and may injure consumers. However, their significant acoustic mismatch with most other food materials makes them relatively easy to detect ultrasonically. Haeggstrom and Luukkala (2001) used ultrasonic signal analysis to detect small pieces of each of these materials at a depth of 20–75 mm in homogeneous materials (margarine, smooth cheese) but to lesser depths (50 mm) in coarser materials (marmalade). In some samples these workers were able to distinguish between the materials based on the ultrasonic echo pattern.

H. POLYMERIC SYSTEMS

The adiabatic compressibility of a solution can be calculated from measurements of its ultrasonic velocity and density (Eqn (7)). The compressibility of a solute is usually taken as the molecular increment of solution compressibility measurements made at very low concentrations. The extremely dilute (∼1 mg ml⁻¹) solutions used in these measurements are extremely advantageous for high value or hard to obtain materials but also require extremely sensitive measurement apparatus. Precise density measurements can be made with a vibrating U-tube densitometer and ultrasonic velocity measurements with a precision resonator. Apenten and coworkers (2000) recently showed that the compressibility of proteins can be measured at relatively high concentrations (>10 mg ml⁻¹) if thermal scattering effects are accounted for. High concentrations means that less-precise pulse echo methodologies may be used.

Compressibility measurements are a useful way to probe polymer, particularly protein, hydration. The measured value is taken to be the sum of contributions from the hydration layer and the intrinsic compressibility of the polymer. Compressibility measurements have been used to reveal
protein hydrophobicity, polar/nonpolar residues, and aspects of secondary structure (Gekko and Hasegawa, 1986) as well as changes associated with protein denaturation (Kamiyama and Gekko, 1997) and ligand binding (Gekko and Yamagami, 1998). Protein–solvent interactions are believed to be important determinants of functionality, and compressibility is unsurprisingly correlated to protease susceptibility, foaming capacity, and free energy of unfolding of a number of proteins (Gekko and Yamagami, 1991).

Gelation and melting of polymer systems has proved a more difficult process to probe ultrasonically. This may be because longitudinal ultrasound is sensitive to the bulk modulus and gelation usually involves changes to the shear modulus. However, in some cases attenuation has proved sensitive to polymer aggregation phenomena, perhaps due to the scattering by aggregates. Gunasekaran and Ay (1994, 1996) used a through-transmission pulsed method to measure the ultrasonic properties (velocity and attenuation) of milk after renneting. They showed that while the changes in ultrasonic velocity were small, the attenuation decreased, perhaps due to the same thermal overlap effects thought to be responsible for the attenuation decrease when an emulsion flocculates. Similar studies on the isoelectric precipitation of other proteins have also revealed a change in attenuation as the proteins aggregated (Pavlovskaya et al., 1992; Bryant and McClements, 1999b). Buckin and Smyth (1999) were concerned with the tendency of calcium-fortified milk to aggregate and gel or precipitate on heating and used a high-precision resonator method to detect the onset of aggregation of their samples. Audebrand and coworkers (1995) demonstrated an increase in attenuation (greater at higher frequencies) as alginate gels. Shore and coworkers (Shore et al., 1986; Shore and Miles, 1988b, 1988c) saw a peak in some muscle preparations (but not in liver or kidney) at pH 5 corresponding to a maximum shortening and density and minimum water-holding capacity of the meat. By suspending the myofibrils in solutions of different density and viscosity they were able to show that viscous scattering was not a significant contributor (~20%) to the total measured attenuation losses (Shore and Miles, 1988a), but they were not able to identify the dominant mechanism. Bryant and McClements used scattering theory to interpret the attenuation spectra of aggregating whey protein in terms of the sizes of structures present (Bryant and McClements, 1999a, 1999b).

Various investigators have seen an ultrasonic attenuation maxima at pH ~ 11 for solutions of amino acids, polypeptides (Appelgate et al., 1968; Saravazyan and Kharakoz, 1979) and proteins (Kanda et al., 1972; O’Brien and Dunn, 1972) owing to the excess absorption caused by the resonance of the protonation equilibrium at amine groups. Miles and others observed a similar attenuation peak at pH ~11 in homogenates (Shore and Miles,
1988c) and suspensions (Shore and Miles, 1988b) of bovine skeletal muscle as well as other beef tissues (liver and kidney). By repeated fractionation of the protein components, they ascertained that this was largely due to a molecular relaxation process rather than to any scattering phenomena. Bryant and McClements (1999b) measured a similar pH-dependent peak in solutions of whey protein isolate and used ultrasonic spectroscopy to estimate the relaxation time of the process (~10^-8 s)

I. MISCELLANEOUS APPLICATIONS

Varadan and Gardner (1999) reported on the use of a Love wave-based smart tongue and nose. Love wave resonance (108.7 MHz) depended on the properties of the materials in contact with the sensor (air, water, orange juice, deicer). Using similar sensors and interpreting the data using principle component analysis, Varadan (personal communication, 2000) was able to distinguish fruit juices (prune, grapefruit, grape, apple, orange) by type.

Vegetable oils deteriorate during frying, breaking down to form off-flavor volatiles and polymerizing to produce a dark brown color and an increase in viscosity. Lacey and Payne (1994) showed that the ultrasonic properties of oil also change with aging, but the correlations are probably too weak to detect spoilage before it becomes obvious.

Withers (1996) considered methods to evaluate pipe fouling and showed that ultrasound can quantify films of thickness between 0.5 and 6 mm (the lower limit being set by the wavelength of the ultrasound). A disadvantage of this method is that it is a single-point inspection and may not be representative of an entire piece of equipment. In non-food applications, Rose (1999) has pioneered the use of guided waves for fouling detection and has shown that a single transducer can inspect several meters of pipe (or kilometers of railroad track).

J. IMAGING

By making several measurements at different positions throughout the food it is possible to construct an image either of the fundamental ultrasonic properties or, by using several of the methods set out above, code the image to composition, temperature, etc. As with all ultrasonic measurements, it is important to remember that the measurements are a function of the composition, microstructure and physicochemical properties of the sample, and converting a time-of-flight into another variable for imaging purposes is not trivial. Pinfield and coworkers (1994, 1996) provide a good example of this problem, demonstrating that the microstructure of
a creaming emulsion as well as the local oil concentration can have an
effect on the measured velocity profile.

A single ultrasonic measurement is analogous to drawing a line (of
finite width) through the body under consideration. The detected signal
will contain information, in terms of echo times and amplitude, about the
structures in its path. By taking many measurements at different positions
on (or angles through) the body, it is possible to provide information about
all the detectable internal structures. The process of reconstructing an
image of these structures from a set of one-dimensional signals is known
as tomography, and while the theory is well understood (Hoyle, 1998) and
commonly exploited in medical (Shung, 1990) and materials imaging, it
has not been adopted in its full form in foods. It is worth remembering that
imaging is a way of generating two-dimensional data from a much richer
data set and is therefore a data reduction operation. The full set of measure-
ments is an A-scan, the image generated from selecting the amplitude at a
set time is a B-scan, and that generated from the amplitude of a selected
signal feature is a C-scan. It is important to bear in mind that both B-scans
and C-scans are data reduction tools and there is often some valuable
information lost in generating an image (Coupland and McClements, 2001a).

Perhaps the most sophisticated imaging regularly used in foods is in the
field of carcass grading. Images can be generated based on a number of ultra-
sonic principles and used to estimate the texture and composition of carcasses,
as well as quality attributes such as marbling and defects (Griffin et al.,
1999; Brethour, 2000). An interesting extension to conventional imaging is the
use of elastography, where an image coded to elastic modulus is generated
by measuring the movement of an ultrasonic signal reflected from the internal
structures in a carcass on gentle bulk compression (Ophir et al., 1994).

Ahvenainen et al. (1989) and Wirtanen et al. (1992) used ultrasonic
imaging to measure the spoilage of milk in cartons (presumably through
the formation of gas bubbles by the active microorganisms present). Acton
et al. (1986) used a medical imaging system to generate some images of
gels. Other workers have simplified the geometry of their system to allow
only one-dimensional changes and recorded images of emulsion creaming
(Howe et al., 1986; Gunning et al., 1989; Dickinson et al., 1994; Basaran
et al., 1998b) and the diffusion of small molecules in gels (Basaran et al.,
1998a) and emulsions (Basaran and McClements, 1999).

Ozguler et al. (1998) measured reflected (backscattered) ultrasonic energy
(17.4 MHz) and used the signal to detect leaks (air- or water-filled channels
and food inclusions) in laminated foil or plastic retortable pouches. The
defects that could be detected were in the order of tens of micrometers.
Frazier et al. (2000) used a modification of this approach to increase the
resolution of the structures imaged.
An important limitation with acoustic imaging is that the sample under investigation must be placed in a tank of water to allow good acoustic propagation from the transducer to and through the food. The measurements are also quite slow and limited by the speed of the robot arm used to position the transducer. (Simultaneous measurements with arrays of transducers can greatly increase the speed of imaging.) These limitations are acceptable for certain high-value items, for example in the aerospace and materials science industries, but it is clearly unacceptable for the routine inspection of foods. However, the use of noncontact, air-coupled transducers offers a way to extend imaging to practical food characterization. Saggin and Coupland (2001c) have shown that noncontact ultrasonic velocity measurements can provide a good description of the size of various food items. For the various soft solids used in this study the ultrasonic measurement of thickness was superior to measurements taken with calipers. A particularly important aspect of the noncontact methodology to size measurement is that the ultrasonic properties of the material are measured simultaneously and do not need to be known \textit{a priori}. A more sophisticated image based on noncontact ultrasonic velocimetry is reported in Figure 15. Noncontact imaging is potentially competitive with NMR imaging currently used in medical diagnostics and, just as NMR imaging has proved invaluable in various types of food characterization, we expect that it will also see wide applications in the food industry.

FIG. 15 Image of a coin generated from the sound reflected from the surface in air-coupled (noncontact) mode. The image was produced using an NCA-1000 (SecondWave Systems, Boalsburg) and an NCT230-R6 (3 MHz, 6.4 mm diameter and 6.4 mm focal length) transducer. (Image courtesy of Dr M. Bhardwaj.)
Scanning acoustic microscopy (SAM) is a useful nondestructive evaluation (NDE) method often applied to the measurement of micro-cracks or other defects in ceramics, composite materials and electronic materials. An important advantage of ultrasonic microscopy over other imaging techniques is in the detection of defects inside opaque materials without requiring sectioning or other destructive operation. Conventionally SAM works in contact mode, detecting reflections of high frequency (tone bursts at 5–230 MHz) sound from the microstructures of interest. The reflected signal is converted to an image dependent on the thickness of the layers and their impedance mismatch. Because of the low impedance of air, delaminations, bubbles and other voids are most easily imaged by this method (Adams, 2000). Aside from the differences in transducer size and frequency, ultrasonic microscopy is similar to other imaging methods and suffers from similar limitations. A couplant material (often water), located between the acoustic lens and the specimen, is usually necessary to allow the propagation of ultrasound into the sample; therefore, the samples are generally immersed in a water tank and repositioned using a stepper motor.

Ultrasonic microscopy has been used to detect sealworms (a parasite) in fish fillets (Hafsteinsson and Rivzi, 1984), but other workers have generated SAM images of biological systems, particularly for medical applications (Kent and Lee, 1997; Kinoshita et al., 1998; Karl and Bereiter-Hahn, 1999).

The resolution of ultrasonic imaging is limited because of the long wavelength of low-frequency ultrasound and the large size of the transducers used. It is possible to modify both of these in a technique known as acoustic microscopy to achieve resolutions approaching an optical microscope. An estimate of the minimum resolvable distance ($\Delta r$) is as follows:

$$\Delta r = F \lambda = F \frac{c_c}{v}$$

(38)

where $F$ is a constant related to the ultrasonic lens geometry and $c_c$ is the velocity of sound in the coupling medium. Therefore increasing the frequency of sound would decrease the resolution of the instrument (for example if 5 MHz sound could resolve points 0.1 mm apart, than 230 MHz sound could resolve points 2 $\mu$m apart). An intriguing alternative method to improve resolution is to use a coupling material with a lower ultrasonic velocity and hence shorter wavelength (for example, if a system could resolve points 0.1 mm apart through a water couplant, then it could distinguish points 0.02 mm apart in an air-coupled system). The air-coupled transducers discussed previously are therefore interesting candidates for high-resolution ultrasonic imaging (Bhardwaj et al., 2000a,
2000b), particularly as the alternatives (liquid nitrogen or helium) are particularly disruptive of delicate structures (Briggs, 1992). It is also important to remember that attenuation increases with frequency so the penetration depth available at high resolution may be limited.

Some recent advances on this technique make use of shear wave lens and noncontact mode, as proposed by Miyasaka and Tittmann (2000). Shear waves offer some great potential, such as a better resolution than longitudinal waves, i.e. they have a shorter wavelength, less sensitivity to surface roughness and lower spherical aberration caused by reflection.

V. CONCLUSIONS

Ultrasound becomes attractive as a sensor in the food industry when it has a unique capability to make a measurement or can otherwise outperform other technologies. Many of the strongest applications gain their advantage from the capacity of ultrasound to propagate through many optically opaque materials, particularly container and piping walls, to make measurements on-line.

The significant disadvantages of ultrasound often center around the fact that propagation depends on a complex set of variables – compositional, structural and dynamic – within the sample and it becomes hard to deconvolute the parameter of interest. Practically, the most significant complicating factors tend to be fluctuations in temperature and/or the presence of air in the sample. The ultrasonic properties of food are strongly temperature dependent and even a 1°C deviation from the calibration conditions can obscure other important changes. The large acoustic mismatch between air and food means that even a tiny amount of entrained gas will dominate the signal or attenuate it to a such a degree as to make measurement impossible. Furthermore, the difficulty of transmitting ultrasound through air limits its application as an on-line sensor for discrete food items.

Another disadvantage is that ultrasonic measurements are often poor multicomponent sensors. Acoustic spectra can be used to identify multiple effects simultaneously but the various relaxations responsible for the changing signal are broad, complex and highly overlapping, so can be difficult to exploit in practice. Velocity and attenuation respond to different properties of the system so are sometimes useful in combination. As a rule of thumb: velocity tends to be more sensitive to composition and temperature while attenuation is more sensitive to structure.

Weighing the advantages and disadvantages, we can see why some ultrasonic applications have seen such a wide application. For example,
the use of ultrasonic attenuation spectra for emulsion characterization allows measurement of a parameter not otherwise accessible (size of concentrated dispersions) under conditions where the other variables (importantly temperature) can be controlled. In cases when the conditions are less well satisfied, the acceptance of ultrasonics is also less, e.g. ultrasonic velocity can be easily used to measure the concentration of sugar syrup in a pipe, but on-line refractive index sensors offer similar functionality and compete for this market. In cases where ultrasound substantially fails to meet the conditions set out above, e.g. multicomponent compositional analysis based upon a temperature scan, we expect limited application.

The successful technologies will be improved and refined through applications by food scientists, but it is also interesting to speculate how this set may be extended by some of the recent innovations discussed in this chapter. Noncontact transducers are particularly interesting as they allow practical application of ultrasonic methods in cases where an air gap between the food and sensor is unavoidable. The use of printed circuit boards to make Love wave sensors that can be wirelessly interrogated has the potential to completely change the economics of sensing. If a fully functional sensor can be made for a few cents, one could be incorporated into each package of food. The use of selective masks for the sensor could allow the detection of spoilage compounds or the metabolic products of microbial growth. Building a reader into domestic refrigerators or cooking equipment could “ask” each piece if it were fresh, safe, or adequately cooked.

No single sensor will meet all the needs of the food industry but, through a critical understanding of both the strengths and weaknesses of ultrasonics, we expect the number of practical applications to grow.

ACKNOWLEDGEMENTS

We are grateful to Dr Mike McCarthy and Y. J. Choi (UC Davies), Dr Mahesh Bhardwaj (Ultran Laboratories/SecondWave Systems, Boalsburg, PA), and Julian McClements (University of Massachusetts) for contributing unpublished data, figures and valuable discussion to this paper, and to Melissa Goff for technical assistance in preparing the manuscript. This work was partly supported by a grant from the Center for Food Manufacturing (Penn State).
REFERENCES


Kippax, P., Sherwood, J. D. and McClements, D. J. 1999. Ultrasonic spectroscopy study of...


OZONE AND ITS CURRENT AND FUTURE APPLICATION IN THE FOOD INDUSTRY

JIN-GAB KIM, AHMED E. YOUSEF and MOHAMMED A. KHADRE

Department of Food Science and Technology
The Ohio State University
Columbus, OH 43210
USA

I. Introduction
II. Ozone Chemistry and Physics: An Overview
   A. Ozone Solubility
   B. Ozone Stability
   C. Ozone Reactivity
III. Medium for Ozone Treatment
   A. Temperature
   B. Relative Humidity
   C. Residual Ozone
   D. Ozone Demand of the Medium
IV. Reactor and Equipment Considerations
   A. Ozone Interaction with Processing Equipment
V. Application of Ozone in Food Processing
   A. Targeted Microorganisms
   B. Inactivation Kinetics and Mechanisms
   C. Ozone as an Alternative Sanitizer in Food Processing
   D. Food Properties and Ozone Applicability
   E. Ozone Applications at Different Stages of Processing
VI. Selected Food Applications
   A. Raw Poultry and Meats
   B. Fruits and Vegetables
   C. Fish Processing and Storage
   D. Dry Food and Food Ingredients
   E. Packaging Material and Food Contact Surfaces
   F. Pesticides on Agricultural Commodities
VII. Combination Treatments
   A. Ozone and Hydrogen Peroxide
   B. Ozone and Chlorine
   C. Ozone and Other Gases
   D. Ozone and Heat
The food industry is interested considerably in using ozone to enhance the shelf-life and safety of food products and in exploring new applications of the sanitizer. This interest was recently accompanied by a US governmental approval of ozone for the safe use, in gaseous and aqueous phases, as an antimicrobial agent on food, including meat and poultry. Ozone has a strong microbicidal action against bacteria, fungi, parasites and viruses when these microorganisms are present in low ozone-demand media. Readily available organic constituents in food, however, compete with microorganisms for applied ozone and thus efficacy of the treatment is minimized. Ozone is suitable for washing and sanitizing solid food with intact and smooth surfaces (e.g., fruits and vegetables) and ozone-sanitized fresh produce has recently been introduced in the US market. Use of ozone to sanitize equipment, packaging materials, and processing environment is currently investigated. Efforts to decontaminate bean sprouts and remove biofilm with ozone have not been successful. The antimicrobial efficacy can be enhanced considerably when ozonation is combined with other chemical (e.g., hydrogen peroxide) or physical (e.g., ultraviolet radiation) treatments. Mechanical action is also needed as a means to dislodge microorganisms from the surface of food and expose them to the action of the sanitizer. The food industry also is interested in using ozone to decontaminate processing water and decrease its chemical and biological oxygen demand. This application improves the reusability of processing water and allows for environment-friendly processing operations.

I. INTRODUCTION

Ozone has been applied industrially for many years, mostly in water treatments, because of its high oxidizing power and superior antimicrobial properties. Use of ozone in the food industry, however, has been limited mainly to shelf-life extension of commodities during storage. Recently,
there has been a renewed interest in ozone and its application in food processing. Application of ozone for decontamination of poultry chiller water seems promising (Sheldon and Chang, 1987; Waldroup et al., 1993; Diaz and Law, 1999) and washing fruits and vegetables with ozone is gradually gaining acceptance. Novel application of this powerful sanitizer will be addressed in this chapter.

Current sanitization technologies are crucial to maintaining the quality and enhancing the safety of fresh agricultural commodities. These technologies, however, have many drawbacks and some treated products are potentially hazardous to consumers. Safety of produce, which is commonly treated with chlorine (or occasionally consumed unsanitized), is currently questionable because of frequent disease outbreaks associated with these products. Pathogens resistant to preservation factors, such as acid-tolerant *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7, have emerged as a serious threat to the fresh produce industry (Beuchat, 1995; Odumeru et al., 1997; NACMCF, 1999). Salmonellosis outbreaks have been associated with pre-cut watermelons and cantaloupes and fresh tomatoes contaminated with *S. Javiana*, *S. Montevideo* and *S. Poona* (Gayler et al., 1955; Ries et al., 1990; Wood et al., 1991; CDC, 1993; *LA Times*, 2001). Water used to wash the tomatoes was implicated as the source of contamination. Outbreaks linked to consumption of unpasteurized apple juice and cider (Besser et al., 1993; CDC, 1996b) also may be attributed to the failure in sanitizing apples properly. Oocysts of *Cryptosporidium parvum*, a zoonotic protozoan parasite, have been detected in unpasteurized cider which caused a disease outbreak (Millard et al., 1994). In 1996, a large epidemic in the United States and Canada involving another protozoan parasite, *Cyclospora cayetanensis*, was epidemiologically linked to raspberries that were imported from Guatemala (CDC, 1996a). Use of nonpotable water for spraying the plant with fungicide was suggested as the source of the pathogen.

In addition to fresh produce, the meat industry can also benefit greatly from new developments in sanitization technology. Meat products have caused numerous foodborne disease outbreaks. *Listeria monocytogenes* has been associated with ready-to-eat meat products resulting in multistate outbreaks of listeriosis in 1998 and 2000 (CDC, 1998, 2000). *Escherichia coli* O157: H7 is traditionally linked to beef products (CDC, 1996c, 1997). This pathogen has caused several disease outbreaks due to consumption of undercooked meat, and the bacterium most likely entered the processing chain on contaminated carcasses.

It is obvious that effective, reliable, economical and industry-relevant alternative sanitization methods are needed. At present, chemical disinfectants such as chlorine and hypochlorites are commonly used as
sanitizing agents in the food industry. Treatments with 50–100 ppm free chlorine solutions reduce initial contamination of vegetables (Carlin et al., 1995), but these levels of the sanitizer may lead to discoloration and production of off-flavors in fresh produce (Hurst and Schuler, 1992). Additionally, chlorination of food could lead to the formation of toxic and carcinogenic chlorinated compounds (Brungs, 1973; Page et al., 1976; Kirk and Mitchell, 1980).

Ozone is an effective, chlorine alternative, sanitizer with superior antimicrobial properties (Kessel et al., 1943; Ito and Seeger, 1980; Korich et al., 1990). It is capable of inactivating bacteria, bacterial spores, molds, yeasts, protozoan cysts and viruses at relatively low concentration and in short exposure time when applied to pure cell suspensions (Giese and Christensen, 1954; Scott and Lesher, 1963; Kim, 1998). Ozone has been tested on nearly every type of food during storage and processing to improve the safety and to extend the shelf-life of these products. The ability of ozone to inactivate contaminant microflora on food is variable; in some instances, however, ozone decreased food microflora more than 5 log units (Yousef and Rodriguez-Romo, 2001). Ozone not only inactivates microbial contaminants, but is also potentially useful in decreasing the level of pesticides, such as azinphosmethyl, captan, formethanate-HCl and ethylenethiourea, on fresh produce (Ong et al., 1996; Hwang, 1999). The chemical oxygen demand (COD) and biological oxygen demand (BOD) of water used in washing and processing of foods can be decreased appreciably by ozonation (Sheldon and Brown, 1986). Thus, use of ozone minimizes the accumulation of inorganic waste in the environment (Horvath et al., 1985). Moreover, rapid decomposition to oxygen and lack of toxic residues make ozone a favorable environment-friendly sanitizer.

Ozone is currently used in many countries and its use in food processing has been approved recently in the United States (Federal Register, 2001). Additionally, ozone-treated produce has just been introduced in the United States market. This chapter addresses current applications of ozone in the food industry, problems that were recently encountered in attempts to apply ozone in food processing, and some probable and challenging future applications. Some of the application problems originated from lack of basic knowledge on sanitization and others are ozone-specific. Recent ozone findings are presented with emphasis on improving the safety of fresh produce.

II. OZONE CHEMISTRY AND PHYSICS: AN OVERVIEW

Ozone is formed in the stratosphere (15–35 km altitude) by the action of short ultraviolet (UV) solar radiation (< 240 nm) on molecular oxygen and
A small portion of ozone is transported to the troposphere (<15 km altitude). About 10% of the atmospheric ozone is present in the troposphere but a very small concentration of ozone occurs naturally at the Earth’s surface (Wojtowicz, 1996). Large amounts of the gas can be synthesized by generators for industrial use. Ozone is a triatomic molecule (O$_3$) that is considered to be an allotropic modification of oxygen. It has a relative molecular mass of 48 and its molecular structure is a resonance hybrid of the four canonical forms having delocalized bonding (Figure 1). Pure ozone is a pale blue gas and bluish liquid with pungent and characteristic odor. Ozone exists in the gaseous state at room and refrigeration temperature and it is partially soluble in water. Ozone has an oxidation–reduction potential of 2.07 V (Brady and Humiston, 1978), which makes it the strongest oxidant currently available for food applications (Table I). The density of ozone in the gaseous state is 2.14 g L$^{-1}$ at 0°C and 101.3 kPa (Wojtowicz, 1996), which is greater than that of air (1.28 g L$^{-1}$) under similar conditions.

![Figure 1](image-url)  
**FIG. 1.** Resonance structures in ozone molecules (Trambarulo et al., 1953).

**TABLE I**  
OXIDATION–REDUCTION POTENTIAL (VOLTS) OF DIFFERENT CHEMICAL OXIDANTS

<table>
<thead>
<tr>
<th>Agent</th>
<th>Molecular formula</th>
<th>Oxidation–reduction potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorine</td>
<td>F$_2$</td>
<td>2.87</td>
</tr>
<tr>
<td>Ozone</td>
<td>O$_3$</td>
<td>2.07</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>H$_2$O$_2$</td>
<td>1.78</td>
</tr>
<tr>
<td>Potassium permanganate</td>
<td>KMnO$_4$</td>
<td>1.70</td>
</tr>
<tr>
<td>Hydrobromous acid</td>
<td>HOBr</td>
<td>1.59</td>
</tr>
<tr>
<td>Hypochlorous acid</td>
<td>HOCl</td>
<td>1.49</td>
</tr>
<tr>
<td>Chlorine</td>
<td>Cl$_2$</td>
<td>1.36</td>
</tr>
<tr>
<td>Chlorine dioxide</td>
<td>ClO$_2$</td>
<td>1.27</td>
</tr>
<tr>
<td>Oxygen</td>
<td>O$_2$</td>
<td>1.23</td>
</tr>
<tr>
<td>Chromic acid</td>
<td>H$_2$CrO$_4$</td>
<td>1.21</td>
</tr>
<tr>
<td>Bromine</td>
<td>Br$_2$</td>
<td>1.09</td>
</tr>
<tr>
<td>Nitric acid</td>
<td>HNO$_3$</td>
<td>0.94</td>
</tr>
<tr>
<td>Iodine</td>
<td>I$_2$</td>
<td>0.54</td>
</tr>
</tbody>
</table>
A. OZONE SOLUBILITY

Ozone is partially soluble in water and its solubility depends on several physical parameters. By Henry’s law, ozone solubility in liquid is directly proportional to the pressure that the gas exerts above the liquid (Bablon et al., 1991). The most important parameter affecting the solubility of ozone is probably water temperature. Based on Henry–Dalton constants, the solubility of ozone in water is higher at lower temperatures. Watson (1943) reported that the solubility ratio (ozone concentration in water phase/ozone concentration in gas phase) was 0.26 at 20°C. Meddows-Taylor (1947) reported a solubility ratio of ~0.4 at 20°C. According to Horvath et al. (1985), the solubility ratio of ozone in water was 0.31–1.13, depending on the water temperature. Our data showed that solubility ratio varied with the source of water used to solubilize ozone. The solubility ratio was 0.16 for distilled water at ~22°C, after ozone was bubbled in the water at 29.4 mL min$^{-1}$ for 18 min (Kim, 1998). When ozone was sparged for 18 min into tap (from two different sources) and deionized water under similar conditions, the solubility of ozone was 38, 56 and 95% of that for distilled water, respectively (Figure 2). Variations in published solubility data may be attributed to differences in the reactor design, gas flow rate and analytical method used to quantitate ozone.

![Figure 2. Treatment of water from different sources with ozone gas (~2.5%, v/v) at a flow rate of 29.4 mL min$^{-1}$ (Kim, 1998). Tap I, tap water in the research laboratory; Tap II, drinking fountain water.](image-url)
High pH interferes with the solubility of molecular ozone. As the pH of ozone solutions increases, the rate of decomposition of molecular ozone into hydroxyl radical also increases (Adler and Hill, 1950; Hewes and Davison, 1973). Researchers attributed the rapid decomposition of ozone in aqueous solutions with high pH to the catalytic activity of the hydroxyl ion. Farooq et al. (1977) noted a greater survival rate of *Mycobacterium fortuitum* during ozone treatment when the pH of the treatment medium was increased. The authors attributed this increased survival to a smaller ozone residual concentration as the pH of water increased. Hydroxide ions are consumed in initiating the ozone decomposition process in water; therefore, ozonation of a solution can decrease its pH value. Sheldon and Chang (1987) found that the pH of poultry-processing water, containing a high organic load, decreased from 6.9 to 5.6 after 50 min ozonation.

**B. OZONE STABILITY**

Ozone is more stable in the gaseous than in the aqueous phase (Stumm, 1958). Stability of dissolved ozone (measured as half-life) is affected by its concentration, pH of the aqueous medium, exposure to UV radiation, presence of radical scavengers (Tomiyasu *et al.*, 1985; Kim, 1998), application of turbulence (Shechter, 1973), temperature (Sease, 1976), and presence of organic matter and metal ions (Horvath *et al.*, 1985). Decomposition of ozone in water does not always follow the first-order rate law (Gurol and Singer, 1982; Tomiyasu *et al.*, 1985; Yurteri and Gurol, 1988). The stability of ozone in water decreases when the pH of the medium increases. High pH is also believed to interfere with the solubility of molecular ozone (Roth and Sullivan, 1981; Ouederni *et al.*, 1987). Kim (1998) found that ozone decomposes rapidly in phosphate buffer when the pH is greater than 8.0 (Figure 3). The researcher bubbled ozone in different types of water and phosphate buffer (pH 7.0) at 25°C, to attain 1.6–2.5 ppm, and monitored ozone decomposition spectrophotometrically. The rate of ozone decomposition was greater in tap water and buffer than it was in distilled, deionized and HPLC-grade water (Figure 4). Ozone stability in the water is greatly influenced by the presence of contaminants, particularly metal ions. According to Bablon *et al.* (1991), however, ionic strength resulting from mineralization of drinking water (< 1000 mg L\(^{-1}\) total dissolved solids) does not affect the solubility of ozone appreciably.

Temperature plays an important role in the stability of ozone in solutions. The half-life of ozone in the gaseous state is approximately 12 h at room temperature and in pure, clean water (pH 7–8) it is 20–30 min (Graham, 1997). The stability of ozone in solutions also depends greatly on the amount of ozone-demand material in the water. Rosenthal (1974)
FIG. 3.  Ozone stability in phosphate buffer having different pH values (Kim, 1998). IC, calculated initial ozone concentration in the ozone–buffer mixture.

FIG. 4.  Ozone decomposition in water from different sources (Kim, 1998). \( N_0 \), ozone concentration initially; \( N \), residual ozone concentration.
C. OZONE REACTIVITY

1. Molecular ozone

Ozone undergoes three types of reactions in organic solvent media (Bailey, 1978): (1) dipolar cyclo-addition with unsaturated carbon–carbon bonds; (2) electrophilic reaction with aromatic compounds, amines and sulfides having strong electronic density; and (3) nucleophilic reaction with carbons carrying electron-withdrawing groups (Figure 5). Therefore, the molecular ozone reactions are selective and limited to unsaturated aromatic and aliphatic compounds as well as to specific functional groups.
2. Products of decomposition – free radical species

Ozone gas is very unstable and decomposes quickly in the air. Because of its instability, the gas is generally produced at the point of application, sparged in water and applied immediately in a closed system. The following discussion addresses ozone decomposition in aqueous solutions. At low concentrations, auto-decomposition of dissolved ozone is an apparent first-order reaction with respect to ozone (Masschelein, 1982; Finch et al., 1988; Peeters et al., 1989). Auto-decomposition of ozone is accompanied by the production of numerous free radical species such as hydroperoxyl (HO₂•), hydroxyl (˙OH) and superoxide (˙O₂⁻) radicals (Adler and Hill, 1950; Hoigné and Bader, 1975). The high reactivity of ozone is attributed to the oxidizing power of these free radicals. According to Jans and Hoigné (1998), when a mole of aqueous ozone decomposes, ~0.5 mol of ˙OH is produced, regardless of whether the transformation is catalyzed by hydroxide anions (i.e. elevated pH), addition of H₂O₂, or exposure to UV

\begin{equation}
\text{HO}_2^- \rightarrow \text{HO}_2 \cdot \rightarrow \text{O}_2^- \rightarrow \text{O}_3^- \rightarrow \text{H}_2\text{O}_2 \rightarrow \text{O}_3 \rightarrow \text{OH}^- \rightarrow \text{HO}_2^-/\text{O}_2^- \text{ are not formed}
\end{equation}

FIG. 6. Ozone decomposition reactions (adapted from Jans and Hoigné, 1998). M, Solute; In, initiator (e.g. OH⁻, HO₂⁻, Fe²⁺, HCOO⁻, –SH, UV); P promotor (e.g. O₃, –SH, R–CH₂OH, Aryl); Ih, inhibitor (e.g. Alkyl, HCO₃⁻, CO₃²⁻, t-BuOH, –SH).
irradiation. The hydroxyl radical is an important transient species and chain propagating radical. The reactions of hydroxyl radical with many substrates are very fast (Hoigné and Bader, 1975). Typical rate constants for reactions of hydroxyl radical with organic solutes are in the range $10^8$ to $10^{10}$ M$^{-1}$ s$^{-1}$ (Farhataziz and Ross, 1977). Ozone decomposition occurs in a chain reaction process (Figure 6) including initiation, propagation, and termination steps (Weiss, 1935; Staehelin et al., 1984; Jans and Hoigné, 1998). The initiators are compounds capable of inducing the formation of the superoxide radical (\(\cdot O_2^-\)); these include hydroxyl ions (OH$^-$), hydroperoxide ions (HO$_2^-$), and some cations and organic compounds (e.g. glyoxylic acid, formic acid and humic substances). Ultraviolet radiation at 253.7 nm also can initiate the free-radical generation process. Promotion reactions regenerate the superoxide radical from the hydroxyl radical. Common promoters include aryl groups, formic acid, glyoxylic acid, primary alcohols and humic acids. Phosphate species are important inorganic promoters. The superoxide anion also can promote the decomposition of ozone. The inhibition reactions lead to the consumption of OH radicals without regenerating \(\cdot O_2^-\). Some of the common inhibitors include bicarbonate and carbonate ions, alkyl groups, tertiary alcohols and humic substances (Hoigné and Bader, 1985). Antioxidants such as tocopherol and ascorbic acid from food also can scavenge the free radicals and thus block the chain reaction.

3. Reactions with inorganic compounds

Minerals, metal ions, hydroxyl ions and halogens (e.g. chlorine) catalyze ozone decomposition and this increases ozone demand (Alder and Hill, 1950; Hewes and Davidson, 1973; Hoigné and Bader, 1976). Reaction of ozone with inorganic compounds found in water usually follows a first-order kinetics, with regard to ozone and the oxidizable compound. Ozone oxidizes ferrous (Fe$^{2+}$) into ferric (Fe$^{3+}$) species, which precipitate in water as ferric hydroxide, Fe(OH)$_3$, and is easily removed by filtration. Similarly, the manganous ion (Mn$^{2+}$) is oxidized by ozone into the manganic (Mn$^{4+}$) state, forming manganic oxide (MnO$_2$), which also precipitates out and can be filtered. These reactions are important for the removal of contaminant metals from drinking water (Dore, 1989). The oxidation–reduction potential values for ozone, chloride, bromide and iodide are 2.07, 1.49, 1.33 and 0.99 V, respectively (Table I). Hence, ozone can oxidize chloride ions slowly, but it reacts moderately with bromide and rapidly with iodide ions, producing elemental bromine and iodine, respectively (Haag and Hoigné, 1983).
III. MEDIUM FOR OZONE TREATMENT

When applied in food processing, ozone gas is used for food storage applications and the aqueous form is used in the surface decontamination of food equipment or packaging materials. Municipal water is commonly used for various washing purposes and dissolution of sanitizers; therefore, this water is the medium of choice for most aqueous ozone applications in food processing. The properties of the treatment medium considerably affect the efficacy of ozone treatment of food. Ozone demand, for example, resulting from dissolved substances in municipal water should be met before the desired sanitizing action occurs. The properties of the medium of significance to ozonation efficacy (i.e. temperature, relative humidity, residual ozone and ozone demand) will be addressed in this section.

A. TEMPERATURE

Several researchers have tested the relationship between ozone efficacy and treatment temperature, but their results seem inconclusive. Kuprianoff (1953) found that ozone was more effective against microorganisms when applied at low (< 10°C) than at high temperatures. Herbold et al. (1989) also reported that the effectiveness of ozone against hepatitis A virus (HAV) and *E. coli* diminished when the temperature increased from 10°C to 20°C. Katzenelson et al. (1974), however, indicated that lowering the temperature from 5°C to 1°C had only a minor effect on the inactivation kinetics of microorganisms. According to Kinman (1975), there is no difference in the disinfection rate by ozone when applied at 0°C or 30°C. Achen and Yousef (2001) treated apples inoculated with *E. coli* O157:H7 in bubbling ozone water at 4, 22 and 45°C for 3 min. The researchers found that residual ozone concentration following the treatments were 36, 22 and 18 mg L⁻¹, respectively, and no significant difference in ozone efficacy between the treatments at three different temperatures was found (*P* > 0.05). The disagreement among researchers may be due to the changes in ozone properties at different temperatures. A decrease in the temperature of an aqueous medium increases the solubility and stability of ozone. On the contrary, an increase in temperature enhances the reactivity of residual ozone. The relative contribution of these two factors (solubility/stability and reactivity) to ozone efficacy may vary with the experimental setup.

B. RELATIVE HUMIDITY

High humidity is needed for inactivation of microorganisms by ozone gas. It is believed that hydration of dry microorganisms in humid atmospheres
makes them susceptible to ozone. The optimum relative humidity (RH) for microbial inactivation by gaseous ozone is 90–95%, and the gas loses its bactericidal effect at ≤ 50% RH (Kuprianoff, 1953). Ozone, however, decomposes more rapidly at high than at low RH values. Elford and van den Ende (1942) used low ozone concentrations and long exposure time at variable relative humidity to disinfect airborne microorganisms. At < 45% RH, the germicidal power of ozone was negligible. Inactivation was substantial at high humidities even when ozone concentration was < 0.1 mg L\(^{-1}\). Ewell (1946) also demonstrated that microorganisms were killed more readily by ozone in an atmosphere having high rather than low RH. Hoffman (1971) indicated that not only were desiccated microorganisms more resistant than hydrated cells to sterilization by ozone, but once desiccated, some cells were difficult to rehydrate sufficiently to be susceptible to ozone sterilization. Ozone, therefore, is an effective sanitizer only against well-hydrated microbial cells. J. G. Kim and A. E. Yousef (Table II, unpublished data) found a similar reaction of ozone in a powdered, food-grade, anticaking agent containing natural contaminants. Application of 200 ppm gaseous ozone caused a minimal decrease in the microbial load of the anticaking agent with a water activity (\(a_w\)) of ≤ 0.84. When an anticaking agent that contained \(a_w\) of 0.96 was treated with 150 ppm gaseous ozone, the microbial load decreased by more than 2 log units. Application of 300 ppm gaseous ozone decreased the microbial load of this agent to an undetectable level. When \(a_w\) of a drier anticaking agent

<table>
<thead>
<tr>
<th>Anticaking agent</th>
<th>(a_w)</th>
<th>pH</th>
<th>Ozone dose (ppm)(^a)</th>
<th>Count (CFU g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.84</td>
<td>8.03</td>
<td>0</td>
<td>(2.7 \times 10^2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200</td>
<td>(1.7 \times 10^2)</td>
</tr>
<tr>
<td>1 (water added)</td>
<td>0.95</td>
<td>8.23</td>
<td>0</td>
<td>(8.2 \times 10^3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>150</td>
<td>(3.0 \times 10^4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>300</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>2</td>
<td>0.96</td>
<td>3.16</td>
<td>0</td>
<td>(5.0 \times 10^3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>150</td>
<td>(1.5 \times 10^4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>300</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>3</td>
<td>0.10</td>
<td>7.00</td>
<td>0</td>
<td>(3.4 \times 10^3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200</td>
<td>(2.3 \times 10^3)</td>
</tr>
</tbody>
</table>

CFU, colony-forming unit.

\(^a\)\(\mu\)g gaseous ozone per g powder.
was increased from 0.84 to 0.95, ozone was as effective in decreasing the microbial load as it was in the product that naturally contained a high water activity. Microbial contaminants in the powder were mostly fungal and bacterial spores, which can survive in a suboptimal growth environment.

C. RESIDUAL OZONE

The term “residual ozone” is used in this chapter to refer to the detectable concentration, in the treatment medium, of ozone after it has been applied to the targeted food product. The effectiveness of ozone against microorganisms depends on the amount applied, but more so on the residual ozone in the medium. The stability of ozone under application conditions and the presence of ozone-demanding material in the treatment medium greatly affect the level of residual ozone available for disinfection of the food product. Venosa (1972) pointed out that one of the most serious failures by various investigators has been their inability to distinguish between the concentration of applied ozone and residual ozone necessary for effective sanitization. Therefore, in addition to the applied dose, the availability and the decay of ozone during the course of the treatments should be reported, otherwise the actual effective dose used may be overestimated.

The results of the following studies illustrate the importance of monitoring residual ozone. Izat et al. (1990) chilled eviscerated broiler carcasses in chlorinated water (20 ppm) or ozonated water at 1.7°C to 4.4°C. The oxidation-reduction potential (ORP) of the control (chlorinated water) was 900 mV and this value decreased as carcasses were introduced into the water. The ozone generator produced 20 g h⁻¹, resulting in an average ORP of 270 mV in the chiller water. After the first 20 carcasses were chilled, total microbial and presumptive coliform counts were significantly higher (unexpectedly) in the ozonated side of the chiller than in the control side. This suggests that either insufficient ozone was generated or the ozone was not remaining in solution for a sufficient time to affect bacterial numbers. In another study, shrimp meat was inoculated with *Vibrio cholerae*, *V. parahaemolyticus*, *Flavobacterium aquatile*, *Pseudomonas aeruginosa*, *P. putida*, *P. fluorescens*, *E. coli*, *Salmonella typhimurium* and *Staphylococcus aureus*. Inoculated meat was immersed in ozonated, 2% saline solution (2.9–4.8 mg L⁻¹ ozone, 5°C) and flushed with ozone at 150 mL min⁻¹ continuously for 60 min (Chen et al., 1992). Ozone concentration decreased by more than 1.4 mg L⁻¹ within 15 min but gradually increased thereafter. Reduction of bacterial count during the first 15 min of flushing was < 1 log unit except for *E. coli*, which had a 2-log unit decrease. A low ozone
concentration, therefore, is ineffective for disinfection of food products when extraneous organic matter is present. Bullock et al. (1997) used ozone to treat water in a recirculating rainbow trout (*Oncorhynchus mykiss*) culture to reduce the heterotrophic bacterial counts in system water and to prevent bacterial gill disease (BGD). Applied ozone was 25 or 36–39 g per kg of feed. Less than 90% reduction of *F. branchiophilum* in water or on gill tissue was achieved but BGD outbreaks were prevented. The limited decrease in bacterial count may be attributed to the short exposure time to ozone (35 s contact chamber) and rapid loss of oxidation caused by suspended organic matter in the medium.

D. OZONE DEMAND OF THE MEDIUM

When compared with other treatment media, pure water has the least ozone demand. Impurities in water react with applied ozone and generate demand. Some of these impurities may initiate ozone decomposition (Hoigné and Bader, 1976); these include glyoxylic acid, formic acid and humic substance. Kim (1998) bubbled several types of water used in the laboratory with ozone gas (1.1 mM) at a flow rate ~ 30 mL min⁻¹ (Figure 2). Ozone dissolved faster in deionized and distilled water than in tap water, resulting in a higher maximum ozone concentration in the former water. The solubility of ozone was >2-fold greater in deionized and distilled water than in tap water. Yang and Chen (1979) reported that the bactericidal efficacy of ozone was lower in Ringer solution, and in solutions containing NaCl (5%) or egg albumin, compared with distilled water. The tested substances decomposed ozone and thus decreased the amounts of residual ozone available for reaction with microorganisms. According to Restaino et al. (1995), death rates of some microorganisms in ozonated water, which contained organic matter, were not significantly affected by 20 ppm soluble starch but were significantly reduced by the addition of 20 ppm bovine serum albumin (BSA). Residual ozone in water containing BSA was significantly lower than in deionized water and water with soluble starch. Achen (2000) varied BSA concentration in *E. coli* O157:H7 suspension and treated the mixture with ozone. The researcher observed no inactivation of the pathogen when >0.1% BSA was added (Figure 7). Ogawa et al. (1990), however, found that addition of 0.5 g loamy soil per liter of ozone solution (1.5–3.8 mg L⁻¹) did not affect the ability of ozone to inactivate spores of *Mucor piriformis*, *Botrytis cinerea* and *Phytophthora parasitica*. Antioxidants originating from food may generate ozone demand by scavenging radicals formed during ozone decomposition. Food additives such as acids, surfactants or sugars can stabilize or destabilize ozone, depending on their properties.
IV. REACTOR AND EQUIPMENT CONSIDERATIONS

For the treatment of food with aqueous ozone, the sanitizer must be transferred from the gas to the liquid phase. The design of treatment chambers and diffusion systems is important to maximize ozone transfer for the intended purpose and to make the process economically feasible. Dissolution/contacting units to provide aqueous ozone for application vary, depending on the specific functions of ozone at the points of application. A number of techniques are available for dissolution of ozone in the liquid. These include conventional fine bubble diffusion, turbine mixers, injectors, packed columns, spray chambers, deep U-tubes, porous plate diffuser contactors, and submerged static radial turbine contactors (Bellamy et al., 1991).

The mass transfer of ozone occurs via diffusion through the gas–water interface. Favorable conditions for ozone mass transfer include high concentration of ozone in the carrier gas and high pressure (Gomella, 1972). According to Henry’s law, high pressure above the process liquid...
increases ozone solubility. White (1986), however, emphasized the importance of contactor efficiency, since maintaining high partial pressures of ozone above the process liquid is sometimes not attainable. Decreasing the diameter of the ozone bubbles increases the total area of exchange and the contact time between water and gaseous ozone (Harris, 1972). Meddows-Taylor (1947) defined “contact value” as the total area of gas bubbles multiplied by the time required to rise a unit distance. The author found that bubbles with a diameter of 0.1 cm have ~ 32 times more contact value than those with a diameter of 1.0 cm. In our experimental work (e.g. Kim and Yousef, 2000b), a sparger with 10 µm pore size was used effectively to decrease the bubble diameter of ozone gas and to increase the transfer rate of gaseous ozone into the water phase. In addition, stirring was carried out during ozonation to ensure sufficient turbulence.

Efficient ozone use in food processing can be achieved by adding a filtration apparatus prior to the contactor. Filtration may keep the level of nontarget demand substances to a minimum and improve ozone dissolution in the contactor (Hampson and Fiori, 1997). Prefiltration of poultry chiller water to decrease the organic load prior to ozone treatment is also recommended for optimum reduction of microbiological levels and efficient use of ozone (Sheldon, 1986; EPRI, 1999). When Sheldon (1986) filtered spent poultry processing water with diatomaceous earth (DE) prior to ozonation, the researcher obtained high-quality water. The filtration–ozonation combination decreased COD, total solids, fats/oil/grease (FOG), total aerobic microorganisms, coliforms and Salmonella spp. by 87%, 65%, 95%, 3 log units, 2.7 log units and 3 log units, respectively. A similar combined treatment was applied on whole-bird rinse water from commercial poultry processing plants (Sheldon and Chang, 1987). Measurable reductions in levels of COD (92%), absorbance at 280 nm (88%), total solids (59%), total volatile solids (82%), aerobic plate count (> 3 log units), coliforms (2 log unit), E. coli (2 log units), and Salmonella spp. (3 log units) were detected following the filtration–ozonation treatment. Filtration was more effective and rapid when DE rather than sand was used in removing contaminants from processing water.

A. OZONE INTERACTION WITH PROCESSING EQUIPMENT

Ozone not only reacts with contaminants (including microorganisms) in the treatment medium and food, it may also interact with the reactor and equipment materials. Efficacy of ozone treatment, therefore, may be influenced by the type of materials used to manufacture the equipment.
1. Reaction with metals

Ozone oxidizes metals except gold, platinum and iridium to oxides of the metals in their highest oxidation states. Ozone converts oxides to peroxides, sulfides to sulfates, carbon to carbon dioxide, and NH$_3$ to NH$_4$NO$_3$. In most oxidation reactions, ozone is reduced to molecular oxygen. Ozone at high concentrations corrodes equipment, but such high concentrations are found only inside the generator or in the ozone-to-water contacting system. Materials used in food processing are usually compatible with ozone at low concentrations (Table III). Stainless steel (e.g. 316L) is corroded less by ozone than by chlorine (Greene et al., 1999; Singh and Singh, 1999). Glass-lined steel (Grosse and Streng, 1960) and steel with a phosphated inside surface (Waller and McTurk, 1965) are

<table>
<thead>
<tr>
<th>Material</th>
<th>Theoretical rating$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>304 stainless steel</td>
<td>Good</td>
</tr>
<tr>
<td>316 stainless steel</td>
<td>Excellent</td>
</tr>
<tr>
<td>Aluminum</td>
<td>Good</td>
</tr>
<tr>
<td>Bronze</td>
<td>Good</td>
</tr>
<tr>
<td>Copper</td>
<td>Excellent</td>
</tr>
<tr>
<td>ABS plastic</td>
<td>Good</td>
</tr>
<tr>
<td>Acetal (Delrin)</td>
<td>Fair</td>
</tr>
<tr>
<td>CPVC</td>
<td>Excellent</td>
</tr>
<tr>
<td>EPDM</td>
<td>Excellent</td>
</tr>
<tr>
<td>Hypalon</td>
<td>Excellent</td>
</tr>
<tr>
<td>Hytrel</td>
<td>Fair</td>
</tr>
<tr>
<td>Kel-F</td>
<td>Excellent</td>
</tr>
<tr>
<td>LDPE</td>
<td>Fair</td>
</tr>
<tr>
<td>Polycarbonate</td>
<td>Excellent</td>
</tr>
<tr>
<td>Polypropylene</td>
<td>Good</td>
</tr>
<tr>
<td>PTFE (Teflon)</td>
<td>Excellent</td>
</tr>
<tr>
<td>PVC</td>
<td>Good</td>
</tr>
<tr>
<td>PVDF (Kynar)</td>
<td>Excellent</td>
</tr>
<tr>
<td>Silicone</td>
<td>Excellent</td>
</tr>
<tr>
<td>Viton</td>
<td>Excellent</td>
</tr>
<tr>
<td>Natural rubber</td>
<td>Severe effect</td>
</tr>
<tr>
<td>Neoprene</td>
<td>Fair</td>
</tr>
<tr>
<td>Nylon</td>
<td>Severe effect</td>
</tr>
<tr>
<td>Buna N (Nitrile)</td>
<td>Severe effect</td>
</tr>
</tbody>
</table>

$^a$Cole Parmer, Vernon Hills, IL.

$^b$Ozone concentration not specified.
resistant to ozone and suitable for maximum half-life storage of ozone. According to a recent report (Viera et al., 2000), ozone in aqueous solution at 0.1 and 0.2 ppm, did not affect containers made of stainless steel and titanium, whereas copper alloys were susceptible to corrosion.

Metals commonly promote the decomposition of ozone and some of these reactions are catalytic. Good catalysts for ozone decomposition include iron, particularly if rusted, zinc, mercury, platinum and silver (Horvath et al., 1985). Large specific surfaces of absorbents such as activated carbon, molecular sieves, silica gel and activated alumina, strongly catalyze the decomposition of ozone (Mahieux, 1962). Greene et al. (1999) reported that pulsing ozone into water at ambient temperature for 20 min per day for 7 days caused greater weight loss of aluminum, carbon steel, copper, 304 stainless steel and 316 stainless steel samples than that observed in the untreated controls; however, weight loss for carbon steel only was significant (P < 0.05). Severe pitting was noted on ozone-treated copper samples when observed by scanning electron microscope. Black striations were observed on ozone-treated carbon steel surfaces. Brass and copper also should be avoided for concentrations > 1.0 ppm aqueous ozone (Hampson, 2000). In an ozone atmosphere of 25–40 mg m$^{-3}$, metal surfaces must be protected by appropriate ozone-resistant painting; however, gas mixtures containing ozone $\leq 5$ mg m$^{-3}$ cause minimal corrosion (Kuprianoff, 1953). The author recommended stainless steel and anodized aluminum for construction of ducts and pipes that carry ozone.

2. Reaction with rubber and plastics

Common plastics used in food processing are generally resistant to ozone (Table II); these include polychlorotrifluoroethylene (EFTFE), polydichlorodifluoroethylene (PDFE), polytetrafluoroethylene (PTFE), polyvinylidenefluoride (PVDF), polyvinylchloride (PVC), and silicon tubing and gaskets (Grosse and Streng, 1960; Mahieux, 1962). Some plastic materials (e.g. PVC and polyethylene, PE) are useful in applications where low ozone concentrations are used. Rubber in seals, pipes and other components reacts actively with ozone, leading to a total disintegration into powder. Synthetic rubbers, however, are resistant to ozone (Horvath et al., 1985). Fluorinated hydrocarbon lubricants have a good resistance to ozone, but polymers of monochlorotrifluoroethylene are the most suitable as lubricants. Silicon grease is adequate for short-term use, but it is oxidized on extended exposure to ozone. The sealing materials on doors and windows of fruit storage rooms should be made of ozone-resistant synthetic materials (Kuprianoff, 1953).
V. APPLICATION OF OZONE IN FOOD PROCESSING

Although ozone is highly effective against microorganisms in pure cell suspensions, it is unlikely to be used directly in food containing high ozone-demand materials. Organic constituents of such food compete with microorganisms for ozone, and thus high doses of this agent may be needed for effective elimination of microorganisms. These high levels of ozone may also alter sensory attributes, and adversely affect the acceptability of food. Current ozone applications in the food industry are mostly related to decontamination of processing water. Ozone, however, has been used with mixed success to inactivate microbial contaminants on meat products, eggs and dry food. Ozone is most suitable for treatment of solid food with intact surfaces such as fresh vegetables and fruits.

A. TARGETED MICROORGANISMS

Gaseous and aqueous ozone, at a low dose and with short contact time, is effective against numerous bacteria, molds, yeasts, parasites and viruses (Kim et al., 1999b; Rodgers et al., 1999). The efficacy of ozone as a sanitizer, however, depends on the target microorganism and treatment conditions. Microorganisms inherently vary in sensitivity to ozone. Ozone was tested recently against Gram-positive vegetative cells, bacterial spores, mold conidiospores and yeast ascospores that are commonly isolated from fruits and known to spoil fruit juices. Results show that bacterial spores are the most resistant and bacterial vegetative cells are the most sensitive to ozone (Kim and Yousef, 2000a). Spores of *Bacillus* spp. varied in susceptibility to ozone (Khadre and Yousef, 2001b). Among eight *Bacillus* spp. tested, spores of *B. stearotherophilus* were most resistant, while spores of *B. cereus* were most sensitive to ozone.

The physiological status of the treated microorganism may affect its susceptibility to ozone. Stationary-phase cells are more resistant to ozone than are cells from the exponential phase. The resistance of microorganism to ozone is greater with natural microflora on food than with microorganisms frequently cultured in the laboratory (Kim et al., 1999a). E. T. Ryser (personal communication) compared the efficacy of sanitizers against natural and artificial contaminants on produce. In produce inoculation studies, ozone (3 ppm) treatments for 5 min decreased the microbial population $\geq 5$ log units. In contrast, naturally occurring background populations generally decreased $\sim 3$ log units after similar treatments. Dormant microbial cells from a dry environment are extremely resistant to gaseous ozone (J. G. Kim and A. E. Yousef, unpublished data). Washing microbial cells
decreases their resistance to ozone because of the removal of ozone-demand material from the cell surface (Schechter, 1973).

Bacteria in biofilms have a polysaccharide architecture that protects them from antiseptics (e.g. hydrogen peroxide and chlorine-releasing preparations) and antibiotics (Dixon, 1998). Microorganisms attached to inert surfaces are less susceptible to the effect of chemical sanitizers than their free-living (planktonic) counterparts (Le Chevaillier et al., 1988). Lethal dose increases when cells have complex envelopes and capsules, specially when cells are in a clump/biofilm. A higher lethal dose is required for spores than for vegetative cells. Existing colonies and clumped cells on the surface of food are hard to destroy because the outer layer of the clump protects inner cells. Mechanical treatment may help break the biofilm structure and increase accessibility of ozone to inner cells (Khadre and Yousef, 2001a).

B. INACTIVATION KINETICS AND MECHANISMS

Ozone kills microbial cells rapidly so that inactivation rates are difficult to measure. Difficulties in obtaining meaningful kinetic parameters have been addressed in a recent publication (Kim and Yousef, 2000b). Uniform procedures to measure inactivation kinetics and establish dose–response plots also were discussed. Suitable indicator microorganisms or spores should be used to measure ozone efficacy. Khadre and Yousef (2001b) suggested *Bacillus stearothermophilus* spore as an indicator of ozone sanitization.

A clearer understanding of the mechanism of inactivation is needed to optimize the effectiveness of ozone and supporting technologies. Ozone activity is likely related to its molecular form (Hunt and Marinas, 1997) or intermediate reactive species such as free radicals and singlet oxygen (Kanofsky and Sima, 1991). It appears that ozone causes damage to the following cellular constituents: (1) unsaturated lipids in the microbial cell envelope; (2) the lipopolysaccharides layer of Gram-negative bacteria; (3) intracellular enzymes; and (4) microbial genetic materials. Earlier studies suggest that ozone reacts with microbial cell membranes (Giese and Christenser, 1954). Ozone is believed to cause the oxidation of lipids on the cell envelope of bacteria (Murray et al., 1965; Scott, 1975). Further oxidation leads to leakage of intracellular cell contents, damage of genetic material and death (Prat et al., 1968; Shechter, 1973). Ozone reacts with cell dehydrogenases (Ingram and Haines, 1949), DNA (Scott, 1975) and RNA (Kim et al., 1980). Khadre and Yousef (2001b) examined spores of *B. subtilis* with the electron microscope after these spores were treated with ozone. The authors observed damage to the outer spore coat layer, which may serve as a primary target of ozone.
Electron microscopic analysis revealed damage to cellular structures after ozone treatment (Kim, 1998; Dave, 1999; Khadre and Yousef, 2001b). Damage was more pronounced in Gram-negatives, *P. fluorescens* and *E. coli* O157:H7, than in Gram-positives, *Leuconostoc mesenteroides* and *Listeria monocytogenes* (Kim, 1998). When treated with ozone under similar conditions, Gram-positive bacteria seemed to lose only some mucoid material outside the cell wall, whereas Gram-negative cells tended to collapse and lose cellular components. Therefore, ozone at low concentrations damages the outer membrane of Gram-negative bacteria and thus causes dramatic changes in cell structure. Similar concentrations of ozone cause less visible damage to the cell wall of Gram-positive bacteria, but the agent causes intracellular damage and effectively inactivates these cells (Kim, 1998).

Kim (1998) also tested the injury of microorganisms by ozone. The degree of injury varied depending on the microorganism, ozone concentration and exposure time. Maximum injury occurred at ozone concentrations that caused mild lethality. Combined treatment of ozone and pulsed electric field (PEF) resulted in a synergistic lethal effect against *E. coli* O157:H7 and *L. monocytogenes* (Unal et al., 2001). It was suggested that the synergy may result from cell injury during the ozone treatment and rapid inactivation of injured cells when they were subsequently treated with PEF.

### C. OZONE AS AN ALTERNATIVE SANITIZER IN FOOD PROCESSING

The food industry has traditionally used chlorine to limit microbial growth on processing equipment and in wash water. Fresh-cut produce is commonly treated with chlorine to extend product shelf-life and minimize the risk of foodborne pathogens. Despite the benefits of chlorine, chlorination may lead to the formation of toxic or carcinogenic chlorinated organic compounds in water (Brungs, 1973; Page et al., 1976; Kirk and Mitchell, 1980) and food, or on food contact surfaces (Wei et al., 1985). Although considerable chlorine-related research has been done to determine optimal parameters for reducing pathogens and extending product shelf-life, its effectiveness on fruits and vegetables remains variable and unpredictable (Nguyen-the and Carlin, 1994). Some studies suggest that chlorine, at high concentrations, causes only modest inactivation of pathogens on food. When Brussels sprouts were treated with 200 mg L\(^{-1}\) chlorine, the count of *L. monocytogenes* decreased by only 2 log units (Brackett, 1987). Beuchat and Brackett (1990) also reported that treating shredded lettuce with chlorine did not prevent the growth of *L. monocytogenes* after the lettuce was packaged in modified atmosphere. Chlorine dioxide treatments only inactivated 1.1 log units of *L. monocytogenes* population on lettuce and 0.4 log unit on cabbage (Zhang and Farber, 1996). To improve the safety
of sprouts, treatment of seeds with 20,000 ppm chlorine has been recommended (US-FDA, 1999).

Given the drawbacks of chlorination, researchers are actively seeking alternatives to chlorine use in food processing. Chlorine dioxide offers several advantages over chlorine as a sanitizing agent and disinfectant (Dychdala, 1991). Based on availability of oxidative species in solution, the oxidation capacity of chlorine dioxide is 2.5 times greater than that of chlorine. Compared with chlorine, chlorine dioxide is also slower in dissociation/hydrolysis, stable over a broader pH range, less corrosive to metal equipment, and less likely to form chlorinated by-products and potential carcinogens. Chlorine dioxide has been used for many years as a water disinfectant because of its bactericidal qualities and its ability to react with humic substances without the formation of trihalomethanes.

Hydrogen peroxide and peracetic acid are potential alternatives to chlorine as a food sanitizer. In a study of various sanitizers for beef brisket adipose tissue, Gorman et al. (1995) found that hydrogen peroxide is one of the most effective sanitizers. While Bundegaard-Nielsen and Nielsen (1996) reported that peracetic acid was not effective as a fungicide, Orth and Mrozek (1989) demonstrated the effectiveness of peracetic acid in reducing numbers of several foodborne bacteria.

Ozone is emerging as a viable alternative to chlorine. The strong germicidal action and the high oxidation potential are some of ozone’s properties that make it an attractive alternative to the traditionally used chemical disinfectants. In addition, ozone decomposes rapidly to oxygen and it leaves no toxic residues; this makes ozone a favorable sanitizer for users concerned about the environment. Ozone, compared with chlorine, is a more powerful and efficient antimicrobial agent against spores, fecal and pathogenic microorganisms, and viruses in an environment containing a high proportion of organic matter (Gomella, 1972). Ozone is also effective for pesticide residue reduction (Ong et al., 1996), food preservation, shelf-life extension, equipment sterilization and improvement of food plant effluents (Horvath et al., 1985; Hampson, 2000). The superiority of ozone over chlorine compounds was reported by many researchers (Kessel et al., 1943; Scarpino et al., 1972; Korich et al., 1990). Of particular importance to the fruit and vegetable industry is a report indicating that greater than 90% of C. parvum oocysts added to water were inactivated after 5 min of exposure to 1 ppm ozone (Korich et al., 1990). In contrast, approximately 90 min of exposure to 80 ppm chlorine were required to achieve similar results. Ozone is effective in surface decontamination of fresh produce. Ozone was recommended recently as an alternative to chlorine (Kim, 1998) and hydrogen peroxide (Khadre and Yousef, 2001b).
D. FOOD PROPERTIES AND OZONE APPLICABILITY

1. Food composition

Commodities with different chemical composition require different ozone dose for effective sanitization. Fresh meat, which contains high fat contents, for example, requires more ozone than do fruits and vegetables, which contain low fat and high carbohydrates. Fournaud and Lauret (1972) treated beef with gaseous ozone during refrigeration and thawing to reduce the surface microorganisms. Gaseous ozone concentrations as high as 500 ppm caused little microbial inactivation. The authors attributed treatment inefficacy to the reaction of ozone with fat and proteins in the meat rather than with the contaminating microorganisms. Kaess and Weidemann (1968b) found that the ozone consumption per unit area of fatty surface tissue was considerably smaller than that of muscle tissue.

2. Surface structure

The nature of the surface of food contributes substantially to the efficacy of ozone treatment. Bacteria on poultry carcasses are located primarily on skin surfaces, within feather follicles and on exposed muscle surfaces. Microorganisms located within the feather follicles are generally protected from the bactericidal action of disinfectants, as shown by relatively small reduction in carcass microbial counts during washing and ozonation (Barnes and Impey, 1968). Bullock et al. (1997) used an ozone treatment in a recirculating rainbow trout (Oncorhynchus mykiss) culture system and prevented bacterial gill disease (BGD) outbreaks but found that the causative bacterium, F. branchiophilum, was still colonizing gill tissues. In recent studies, bubbling ozone in wash water for 3 min was effective in reducing microbial counts on the surface of apples by up to 3.7 log units (Klingman and Christy, 2000; Achen and Yousef, 2001). Ozone treatment, however, was less effective in decontaminating the calyx and stem areas of the apple. Spraying water on these areas prior to the ozonation helped dislodge the cells and reduced the counts by 1.5 log CFU g⁻¹. When E. coli O157:H7 was permitted to attach to the apple surface, the efficacy of disinfection by ozone diminished.

In conclusion, strongly attached surface microorganisms and those attached to areas that are not freely exposed to ozone cannot be eliminated by mere dipping in ozonated water. In addition, microorganisms embedded in product surfaces are more resistant to ozone than those suspended in water. Therefore, when ozone is applied in food processing, good contact between the sanitizer and the target microorganisms on the treated food...
should be ensured. A variety of methods have been used to accomplish this goal including stirring, pumping, fluming, bubbling, sonication, abrasion and pressure washing.

3. Release of exudates

Most fruits and vegetables have a hard protective layer of peel, skin or rind, and the outer surface is usually covered with a waxy material. These products commonly have a limited ozone demand. However, in minimal processing, fresh vegetables and fruits are usually trimmed, peeled (or cut if necessary) and washed, thus tissues are exposed and cellular fluids are released that increase ozone demand. When hot water or steam is used for blanching, vitamins, flavors, colors, carbohydrates and other water-soluble components are inevitably leached (Ihl et al., 1998); this released organic matter constitutes ozone-demand. Treating meat with ozone is a high ozone demand process, since the meat surface has crevices and it leaches ozone-demanding organic substances such as fats and proteins.

4. Injuries and wounds

Gaseous ozone treatment was not effective in decreasing infection in inoculated wounds in apples (Schomer and McColloch, 1948). Ogawa et al. (1990) readily inactivated spores of B. cinerea on the surface of non-injured tomato fruit using 3.8 mg L\(^{-1}\) aqueous ozone for 10 min. However, spores placed on the surface of injured tomato fruit were not inactivated. Smock and Watson (1941) reported that when spores were protected by moist surfaces of apple flesh or by other organic protectants, ozone had no effect on their germination. When pear fruits were wound-inoculated with Penicillium expansum and then treated with 5.5 mg L\(^{-1}\) aqueous ozone for 5 min, levels of decay were similar to those of a control treated with water only (Spotts and Cervantes, 1992). This suggests that plant tissues and extracellular biochemicals at wound sites react with ozone and render it ineffective.

E. OZONE APPLICATION AT DIFFERENT STAGES OF PROCESSING

Ozone may be applied directly on raw agricultural commodities at the pre-processing stage, during processing, or on the finished product while at storage. It is usually advantageous to apply ozone on the raw than the processed product. Whole grains, for example, require less ozone to disinfect than does the powder product (Naitoh et al., 1987). Raw solid food commonly has intact surface and most of the natural contaminants are limited
to the surface. Elimination of readily accessible surface contaminants is feasible using most sanitizers including ozone. Sanitizers, however, may differ in dealing with contaminants that are attached or embedded in food surfaces. Aqueous ozone can be used to decontaminate beef and beef brisket fat (Gorman et al., 1995), poultry meat (Dave, 1999), salmon (Goche and Cox, 1999), apples (McLoughlin, 2000; Achen and Yousef, 2001), strawberries (Lyons-Magnus, 1999), lettuce (Kim et al., 1999a), broccoli and cauliflower (broccoflower) (Hampson and Fiori, 1997) and other commodities. Microbial studies typically show a reduction of 2 log total count and a significant reduction of spoilage and potentially pathogenic species that are most commonly associated with fresh fruits and vegetables. When these raw foods carry high organic loads, effectiveness of ozone treatment is likely to diminish. Multiple-stage wash system may be needed in this case with a pre-wash and a rinse preceding the ozone treatment.

Some researchers used ozone to treat ingredients before they are included into food formulation. Kim et al. (1993) treated various spices, used to prepare Kimchi, with ozone and improved the fermentation of the final product. J. G. Kim and A. E. Yousef (unpublished) also used ozone to decontaminate the ingredients of fruit juices such as high-fructose corn syrup. These researchers speculated that ozone treatment of ingredients, rather than the final juice, reduces ozone usage and minimizes damage to the sensory quality of the final product. Naitoh et al. (1989) reported that the treatment of wheat flour with ozone inhibited microbial growth in namamen products and increased their storage life.

Application of ozone in the processing facility to minimize environmental contaminants has been studied (Naitoh, 1993; Hampson, 2000). Combinations of ozone with other oxidants such as hydrogen peroxide were used to sanitize packaging films (Gardner and Sharma, 1998), a confectionary plant (Naitoh, 1989), and hatchery equipment (Whistler and Sheldon, 1989). Ozone decreased surface flora by ~3 log units when tested in wineries for barrel cleaning, tank sanitation, and clean-in-place (CIP) operations (Hampson, 2000). Some wineries have embraced the use of ozone for multiple purposes, including barrel and tank cleaning and sanitation, CIP systems, and for general purpose sanitation (Duca, 1999; Hampson, 2000). Aqueous ozone, at 1.5 ppm, was tested in a food-processing facility. The treatment decreased the microbial load on a stainless steel kettle, table top, shroud, plastic shipping container, floor surface and drain (Hampson, 2000). The author reported reductions of up to 3 log total plate count on floor surfaces and shrouds, but less than 1 log unit in floor drains. The author recommended using ozone in a complementary sanitizing regime to maintain the overall cleanliness and sanitation of wineries and any other food-processing facility.
Gaseous ozone can be used to extend the shelf-life of food during storage. Ozone minimized growth of surface contaminants on meat (Greer and Jones, 1989), grapes (Sarig et al., 1996) and broccoli florets (Zhuang et al., 1996) when the gas was applied during storage of these commodities.

VI. SELECTED FOOD APPLICATIONS

A. RAW POULTRY AND MEATS

Count and diversity of the microbial population dictates the shelf-life of raw poultry and meats. In addition to numerous spoilage microorganisms, these products occasionally carry pathogenic microorganisms such as Campylobacter spp., Salmonella spp., L. monocytogenes and pathogenic E. coli. The types and number of microorganisms present on raw poultry and meats depend on the microorganisms that colonize the gastrointestinal tract. Food contamination with these microorganisms can occur at multiple steps along the food chain including slaughtering, handling, storage and distribution (Zhao et al., 2001). Use of sanitizers on carcasses and cut meat is currently limited. Chlorine may be used in the poultry chiller tanks or as spray on carcasses, but alternative and effective sanitizers have been pursued.

Several investigators tested decontamination of beef and beef brisket fat by ozone; results were variable (Gorman et al., 1995, 1997; Reagan et al., 1996). Other research groups found that gaseous ozone minimized or prevented growth of microorganisms on the meat surface (Kaess and Weidemann, 1968a; Greer and Jones, 1989; Mitsuda et al., 1990). Numerous investigators have demonstrated the microbicidal efficacy and safety of ozone for use in washing poultry carcasses (Barnes and Impey, 1968; Yang and Chen, 1979; Sheldon and Brown, 1986; Caracciolo, 1990; Izat et al., 1990; Jindal et al., 1995), reconditioning poultry chiller water (Sheldon, 1986; Sheldon and Chang, 1987; Waldroup et al., 1993; Diaz and Law, 1999), and sanitizing hatchery equipments (Whistler and Sheldon, 1989). Advanced oxidation processes, including ozone and adjuncts such as hydrogen peroxide and UV radiation, enhanced the efficacy of ozone as an antimicrobial control agent in poultry chiller water (Diaz and Law, 1999; EPRI, 1999). Prefiltration of the chiller water prior to ozone treatment is recommended for optimum reduction of microbiological levels and efficient use of ozone (Sheldon, 1986; EPRI, 1999).

The presence of Salmonella enterica ser. Enteritidis in shell eggs has serious public health implications. Several thermal and chemical treatments have been developed to control or eliminate this pathogen in
eggs. Yousef and Rodriguez-Romo (2001) used ozone at low temperatures and under mild pressure for cold sanitization of shell-eggs. Shell-eggs were externally contaminated with *S. Enteritidis* so that shells contained $\sim 10^6$ CFU g$^{-1}$. Eggs were treated with gaseous or liquid ozone for 1–20 min, at 4–25°C and 0–15 psi (0–100 kPa). Gaseous ozone treatment without pressure decreased the count of *S. Enteritidis* on shells by 2.2–2.7 log units. Treating contaminated eggs with gaseous ozone for 10 min at 22–25°C and 15 psi decreased *Salmonella* population by more than 5 log units. Such a treatment may be used industrially to produce “cold-sanitized” eggs. Cox *et al.* (1995) patented a “hyperpasteurization” process, which uses vacuum, heat and ozone, to eliminate *Salmonella* spp. from the contents of shell eggs. This method includes, heating shell eggs at higher than 54.4°C for longer than 15 min with subsequent application of ozone. According to this report, the combined treatment extended the shelf-life and reduced the microbial load of shell eggs.

B. FRUITS AND VEGETABLES

There are many steps in the food production chain with multiple potential sources of contamination at each step. For example, dirty irrigation water, manure fertilizer, and improper worker hygiene have been cited as probable causes for pre-harvest contamination of fresh fruits and vegetables. Following harvest, improper handling and storage, use of contaminated wash water, processing equipment and transportation facilities as well as cross-contamination from other produce contribute to the microbial hazards associated with fresh fruits and vegetables. Of special concern is the quality of wash water and the potential hazard associated with cross contamination (Tauxe *et al.*, 1997). Following cutting, shredding and slicing of fresh-cut fruits and vegetables, the loss of surface integrity can lead to penetration and rapid growth of microorganisms. In a study on processing conditions of chopped lettuce and coleslaw, shredders were identified as the major source of in-plant contamination (Garg *et al.*, 1990). Other contributing factors include the expansion of production facilities and longer food marketing chains that allow the distribution of more heavily contaminated produce to wider populations (Tauxe *et al.*, 1997). Refrigerated fresh-cut produce is susceptible to microbial spoilage (Nguyen-the and Carlin, 1994) and growth of pathogenic microorganisms (Beuchat, 1995). Ozone application for improving the safety and extending the shelf-life of fresh-cut produce seems feasible. Improving water reusability by the fruit and vegetable processing industry is an additional benefit of ozone application. Ozone-sanitized produce has been introduced recently in the United States market after several years of developing and testing (TVA,
The following are examples of studies and successful attempts to sanitize fresh produce with ozone.

1. Apples

Apples are subject to contamination with pathogenic microorganism on the farm. Use of contaminated apples to produce unpasteurized apple juice and cider resulted in *E. coli* O157:H7 foodborne infections (Besser *et al.*, 1993; CDC, 1996b). The Food and Drug Administration (FDA) now regulates the production of cider with recommendations for the pasteurization of apple cider and other juice products or the use of alternative processing steps to reduce the counts of the pathogen in question by 5 log units (FDA, 1998). Use of effective sanitizers on whole apples prior to pressing is a feasible option. Chlorine and hydrogen peroxide with surfactants and isothiocyanate have been investigated as sanitizers (Beuchat *et al.*, 1998; Lin *et al.*, 2000). In a recent study, Achen and Yousef (2001) inoculated apples with *E. coli* O157:H7 prior to treatment with an aqueous solution containing 21–28 mg L\(^{-1}\) ozone. Decontamination treatments were more effective when ozone was bubbled during apple washing than by dipping apples in pre-ozonated water. Maximum decreases in surface counts of *E. coli* O157:H7 were 2.6–3.7 log units, compared to unwashed inoculated controls. However, counts of *E. coli* O157:H7 decreased by less than 1 log unit in the stem-calyx region by the ozone treatment. Rinsing the apples with an inorganic wetting agent (tetrasodium pyrophosphate) increased the efficacy of the ozonation process. The wetting agent may have enhanced the contact between ozone and bacterial cells that are attached to the hydrophobic surface of the apple, decreased cell attachment on the stem and calyx areas, and assisted in exposing entrapped cells to ozone. The authors speculated that in conventional apple washing environments, the efficacy of ozone against microbial contaminants may become limited because of the high organic loads in the washing tanks resulting from debris, soils, and fruit saps; these contaminants impose an ozone demand.

2. Lettuce

Kim *et al.* (1999a) tested ozone against natural contaminants in fresh lettuce and results were compared with those obtained from chlorine treatment. Ozone (1.3 mM) or chlorine (1 mM) inactivated mesophilic and psychrotrophic bacteria by 1.4 and 1.8 log units in 3 min, respectively. Counts of these microorganisms on lettuce, from a different batch, decreased 3.9 and 4.6 log units, respectively, during 5 min of ozone treatment. In another
experiment, shredded lettuce was treated with gaseous ozone, or mixed with aqueous solution of ozone (1 : 20 w/w) with or without bubbles. Results show that bubbling gaseous ozone in water, combined with stirring, is the most effective ozonation method for shredded lettuce.

3. Alfalfa sprouts

Alfalfa sprouts received great attention in recent years owing to the incidence of pathogens in this product and associated disease outbreaks. In 1998, the FDA issued a statement warning consumers of high-risk groups to avoid consumption of sprouts due to the potential health hazard associated with these products. J. M. Boff and A. E. Yousef (unpublished data) determined the effectiveness of ozone in reducing the natural flora on alfalfa sprouts. Treatment of alfalfa sprouts with ozone for 5 min decreased their microbial load by 1.2 log units. An additional 0.8 log unit decrease in population was observed when ozonation was accompanied with agitation. As an alternative ozonation process, alfalfa seeds were pretreated with ozone and the microbial count during seed germination and growth was monitored. Additionally, ozonated water was used to water the sprouts twice daily during the growth period. The initial microbial count on the seeds was ~ $10^5$ CFU g$^{-1}$ and ozone treatment decreased the count by ~ 2 log units. The difference in count between ozone-treated and nontreated seeds diminished during the growth period and the population in both treatments reached ~ $10^9$ CFU g$^{-1}$ after 4 days of seed incubation. Most of the growth of the natural flora occurred in the first 2 days after setting. Treatment with ozone water during the growth period only temporarily decreased the count, but the counts after 4 days of growth were identical in ozone-treated and nontreated sprouts.

An end treatment consisting of bubbling ozone into the sprouts for 2.5 min decreased the microbial count from $5.0 \times 10^9$ to $2.0 \times 10^7$ CFU g$^{-1}$. Alternatively, the sprouts were placed in ozonated water (30–32 ppm) and stirred for 20 min; the average count decreased to $4.8 \times 10^7$ CFU g$^{-1}$. The latter method may be preferable to the former one because of better quality and texture of the resulting sprouts. In conclusion, ozone treatments, as tested in this study, are not sufficient to bring about a substantial reduction in the microbial population on sprouts.

4. Fruit juice ingredients

Technological advances in citrus processing led to development of convenient, shelf-stable, ready-to-drink juices. Survival of heat-resistant bacteria and fungi during pasteurization of juice can be a serious concern
to citrus processors. Ozone is potentially useful in inactivating heat-resistant spores in juice components with low ozone demand. Therefore, the efficacy of ozone against spores of Alicyclobacillus acidocaldarius, Neosartorya fischeri and Zygosaccharomyces bailii, which are known to be problematic in juices, was tested in selected juice ingredients (Kim and Yousef, 2001). Fruit juice components, high fructose corn syrup (HFCS), orange juice concentrate (OJC), and pineapple juice concentrate (PJC), spiked with cells or spores (c. $10^7 \text{mL}^{-1}$), were treated with gaseous ozone. The sensitivity of spores to ozone varied depending on the type of spores and the juice component. The ozone dose required for inactivating 5 log units of A. acidocaldarius spores was 0.31, 0.28 and 0.41 mg ozone per mL spore suspension in HFCS, OJC and PJC, respectively. The ozone dose for inactivating 5 log units of N. fischeri spores was 0.12–0.51 mg mL$^{-1}$, depending on the juice ingredient. The amount of ozone required to inactivate N. fischeri spores was four times greater for PJC than for HFCS. In order to achieve a decrease of 5 log units of Z. bailii spores, 0.04–0.24 mg mL$^{-1}$ was needed, depending on the juice component. Inactivation of Z. bailii spores by ozone was faster in HFCS than in other juice components even though HFCS has higher solids than the other components. In conclusion, the ozone dose required to achieve a 5-log unit decrease of the targeted microorganisms varied from 0.04 to 0.5 mg mL$^{-1}$ with A. acidocaldarius, and N. fischeri spores were three to four times more resistant to ozone than were Z. bailii spores. The authors do not recommend applying ozone directly to juice concentrates or reconstituted juice. Ozone, however, may be safely applied to the HFCS. Ozonation can also be combined with different inactivation technologies to minimize the changes in product quality while maximizing the inactivation of contaminants.

C. FISH PROCESSING AND STORAGE

Ozone was tested for decontaminating shrimp (DeWitt et al., 1984), mussels (Abad et al., 1997), and various fish such as jack mackerel (Haraguchi et al., 1969), sockeye salmon (Lee and Kramer, 1984), Japanese flounder (Mimura et al., 1998) and rockfish (Kötters et al., 1997). The antimicrobial efficacy of ozone was equal to or better than that of chlorine in some applications studied (Arimoto et al., 1996; Goche and Cox, 1999). Ozone reduced disease incidence in hatcheries with less mortality and shorter growth cycles (Błogosławski et al., 1993; Arimoto et al., 1996; Bullock et al., 1997). The absence of adverse sensory effects and harmful oxidation by-products confirms the desirability of ozone use in processing fish products for human consumption. Ozone treatment of shrimp meat extract, however, was ineffective against microorganisms in the product (Chen et
Ozone may have reacted with ozone-demand substances in the extract, instead of microbial cells.

**D. DRY FOOD AND FOOD INGREDIENTS**

Gaseous ozone can eliminate *Bacillus* spp. and *Micrococcus* spp., which are dominant in cereal grains, peas, beans and spices, by up to 3 log units, depending on concentration, temperature and relative humidity (Naitoh *et al.*, 1988). The surface area of the dry food is an important factor for the effectiveness of ozone treatment. Cereal flour and ground pepper, for example, require higher concentration of ozone and longer contact time than whole cereal and pepper to achieve the same degree of microbial inactivation (Naitoh *et al.*, 1989; Zagon *et al.*, 1992). In addition to anti-microbial effects, ozone destroys or greatly reduces aflatoxins from peanut (Dollear *et al.*, 1968) and cottonseed meal (Rayner *et al.*, 1971) and oxidizes odors produced during dehydration of onions and garlic (McGowan *et al.*, 1979). Ozone treatment may cause lipid oxidation (Naitoh *et al.*, 1988), decrease amino acid (e.g. thiamine) content (Naitoh *et al.*, 1989) and essential oil content (Zhao and Cranston, 1995), and contribute negative effects on the sensory quality of some dry food.

J. G. Kim and A. E. Yousef (Table II, unpublished data) used ozone to inactivate natural contaminants in a silica-based anticaking agent. High water activity with limited organic matter provided suitable conditions for growth of bacteria and fungi in this product. Ozone gas at 30–40 ppm was sufficient to sterilize an anticaking agent but failed to decontaminate other types of the substance. The authors hypothesized that water activity is an important factor for cell inactivation by ozone in the anticaking agent.

**E. PACKAGING MATERIAL AND FOOD CONTACT SURFACES**

Microbial contaminants on food packaging materials are commonly small in number, but they may survive conventional decontamination processes and cause food spoilage. Currently, sterility of packaging materials is achieved by several methods including heat, hydrogen peroxide and UV radiation (Stefanovic and Dickerson 1986; Yokoyama 1990; Gardner and Sharma 1998). Sterilization by hydrogen peroxide is a tedious and variable method (Yokoyama 1990), and unacceptable levels of hydrogen peroxide residues may remain and interact with some of the polymer in the packaging material (Stefanovic and Dickerson 1986; Castle *et al.* 1995). Sanitization of equipment and food-contact surfaces is essential for safety and quality of processed food. When biofilms develop on wet food contact surfaces such as those made from stainless steel, the micro-
organisms inside the biofilms are usually protected from sanitizers (Frank and Koffi, 1990; Carpentier and Cerf, 1993; Dixon, 1998). Hence, alternative methods for decontamination of packaging materials and stainless steel are being sought.

A multilaminated aseptic food packaging material and stainless steel were treated with ozone to inactivate natural contaminants, bacterial biofilms (*P. fluorescens*) and dried films of *B. subtilis* spores (Khadre and Yousef, 2001a). Sterility of the multilaminated packaging material, which contained a low natural, mostly mesophilic, contaminants was achieved when it was treated with 5.9 mg L\(^{-1}\) ozone in water for 1 min. Counts of bacteria in dried films decreased by 4.6–5.1 log units for the multilaminated packaging material and 5.5 log units for stainless steel when treated with 5 mg L\(^{-1}\) aqueous ozone for 1 min. Dried films of spores (10\(^8\) per 6.3 cm\(^2\) surface) decreased below detection (< 10 spores per 6.3 cm\(^2\) surface) by application of 13 mg L\(^{-1}\) aqueous ozone for the multilaminated packaging material and 8 mg L\(^{-1}\) in the case of the stainless steel. Repeated exposure to ozone and agitation during the treatment were needed to decrease effectively the population in biofilms. Ozone inactivated *P. fluorescens* in biofilms more effectively on stainless steel than on the multilaminated packaging material. The relatively low efficiency of ozone against bacteria in the biofilm, compared to that in dried films, is probably due to the tenacious adherence of bacteria to the surface of packaging material when biofilms are formed. It is concluded that ozone is an effective sanitizer with potential applications in the decontamination of packaging materials and equipment food-contact surfaces. Removal of biofilms by ozone, however, requires additional mechanical action during the treatment.

**F. PESTICIDES ON AGRICULTURAL COMMODITIES**

Use of pesticides on fruits and vegetables is crucial for insuring high-quality products. Residues of these pesticides, however, raise the concern of consumers and effective means to remove these residues are sought. The use of chlorine, ozone, chlorine dioxide and peracetic acids as postharvest treatments has been effective in remediating several different pesticides on apples (Ong *et al.*, 1996; Siler, 1998; Hwang, 1999). These same treatments also have the added benefit of reducing microbial populations on the surface of fruits and vegetables by up to 5 log units (Rodgers *et al.*, 1999). Organic food processors and consumers of organic food also are concerned about the presence of chemical residues, including chlorine and chlorinated by-products. However, these residue levels are likely reduced by ozonation in solution.
VII. COMBINATION TREATMENTS

Molecular ozone, or free radicals resulting from its degradation, interact with pollutants or microorganisms and cause their destruction. Direct reaction of ozone with organic compounds is generally relatively slow. Therefore, most ozone reactivity is associated with the radical-chain reaction rather than with the direct reaction with solutes (Hoigné and Bader, 1979). Typical rate constants for reactions of the hydroxyl radical with organic compounds are in the range $10^8$ to $10^{10}$ M$^{-1}$ s$^{-1}$ (Farhataziz and Ross, 1977). Thus, microorganisms are inactivated faster in the presence of radicals formed during ozone decomposition than by ozone molecules themselves because of the higher reaction rates of the former.

In order to improve ozone action, free radical generation and the combination of ozonation with other technologies have been studied. Advanced oxidation process techniques are designed to promote the formation of hydroxyl free radicals, resulting in increased microbicidal activity above that of ozone itself. Effective ozonation procedures are also being developed through improved delivery systems in order to overcome the physical barriers that diminish the efficacy of sanitization of products, and to maximize the biocidal action of ozone.

A. OZONE AND HYDROGEN PEROXIDE

The combination of ozone and hydrogen peroxide in aqueous solution generates hydroxyl free radicals (Figure 6). Combinations are obtained by adding the necessary amount of hydrogen peroxide to the water being treated and then passing the solution through an ozone-contacting apparatus (Graham, 2000). Hydrogen peroxide is a weak acid, which partially dissociates into hydroperoxide ion in aqueous solutions.

$$H_2O_2 + H_2O \rightarrow HO_2^- + H_3O^+$$

$$O_3 + HO_2^- \rightarrow \cdot OH + \cdot O_2 + O_2$$

The hydrogen peroxide molecule reacts slowly with ozone, whereas the hydroperoxide anion is highly reactive (Taube and Bray, 1940). Consequently, the rate of ozone decomposition by hydrogen peroxide increases with increasing pH. The reaction rate of ozone decomposition with $H_2O_2$ is theoretically 100 000 times greater than that of ozone decomposition initiated by hydroxyl ions. Therefore, very low concentrations of $H_2O_2$ are kinetically effective in initiating $O_3$ decomposition.
For optimum oxidative performance, a specific weight ratio of peroxide to ozone is required to destroy each pollutant. For example, the taste- and odor-causing compounds, geosmin and 2-methylisoborneol in potable water, require a peroxide to ozone ratio of about 1 : 0.3. Pesticides sometimes require weight ratios as high as 1 : 0.8. Therefore, it is advisable to determine experimentally the optimum peroxide to ozone ratio that is most suitable for a given application (Graham, 2000). Glaze et al. (1987) found that the rate of oxidation of organic load in water decreases when the weight ratio of peroxide to ozone is greater than one. The Electric Power Research Institute (EPRI, 1999) evaluated the efficacy of ozone and hydrogen peroxide combinations in poultry chiller operation. When a broiler was rinsed with chiller overflow ultrafiltrate containing 1–2 mg L\(^{-1}\) ozone and 0.5 mg L\(^{-1}\) hydrogen peroxide, the average decrease in total count was more than 2 log units.

B. OZONE AND CHLORINE

Processors of fresh-cut produce maintain a minimum chlorine concentration in wash water to ensure the efficacy of the treatment. Therefore, chlorine residues are monitored as a critical control point in these processes. Processors who look for ozone as an alternative to chlorine treatments are concerned that the efficacy of sanitization cannot be similarly measured, since ozone treatments may not result in measurable residues.

The potential interaction of ozone and chlorine should be considered when combining these two sanitizers in a processing line. Ozone oxidizes residual chlorine in water to form chlorate and perchlorate, which are weaker oxidants (Kolle, 1968; Siddiqui, 1996). However, Buydens and Fransolet (1971) did not notice any interaction of ozone and chlorine. Therefore, sequential application of ozone and chlorine is potentially a useful combination treatment in such facilities.

C. OZONE AND OTHER GASES

Tahoe Food Technology, Inc. (1998) invented an apparatus that produces a continuous stream of mixed gas containing ozone, carbon dioxide and argon. Navel oranges were treated with this gas mixture for 2 h in a sealed chamber to control bean thrips, red scale and fuller rose beetle at 20°C, 30% RH and 9.5 psi (66 kPa). The concentrations of ozone, carbon dioxide and argon were 4.0, 10.0 and 1.0%, respectively. All adults, larvae and eggs (fuller rose beetle only) were killed in the process. The treated naval oranges were incubated for 28 days after treatment to ensure that all three life cycles had been destroyed.
Mitsuda et al. (1990) tested the decontamination of fresh cucumbers with a mixture of ozone and carbon dioxide gases in polyvinylchloride film bags. The concentration of ozone gas generated was 20–40 g m\(^{-3}\). The food in each bag was exposed for 5 min to ozone, carbon dioxide, and their mixture. The best results were obtained when mixing ozone and carbon dioxide at 3:1 and 2:1 (v/v). After 14 days storage, ~1 log unit decrease of bacteria was obtained with 2:1 and 1:1 mixtures. The survival of microorganisms during storage was lower for the mixed gases of ozone and carbon dioxide than it was for the individual gases. The authors reasoned that this synergistic action was a result of the quenching effect of carbon dioxide on the chain decomposition reaction of ozone, and by the bacteriostatic effect of carbon dioxide.

D. OZONE AND HEAT

Ozone degrades rapidly with heat; therefore, simultaneous application of these two preservation factors is ineffective against food microflora. Sequential application of ozone and heat, however, may be beneficial. J. G. Kim and A. E. Yousef (unpublished data) applied sublethal concentrations of ozone to *B. subtilis* spores and then measured thermal inactivation of pre-treated spores. Results (Table IV) show that ozone treatment greatly sensitized bacterial spores to heat. The D-value was decreased considerably by the sublethal ozone pre-treatment. Khadre and Yousef (2001b) examined ozone-treated bacterial spores using the transmission electron microscope. The authors found that treatment of spores with aqueous ozone (10 mg L\(^{-1}\)) for 1 min caused substantial damage to the outer coat layer. Therefore, weakening of the outer coats by sublethal ozone concentrations may have sensitized *B. subtilis* spores to heat.

E. OZONE AND ULTRAVIOLET RADIATION

An advanced oxidation process, based on a combination of ozone and UV radiation, enhances the antimicrobial action of ozone (Diaz and Law, 202 J.-G. KIM ET AL.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>85°C</th>
<th>90°C</th>
<th>95°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>294</td>
<td>74.6</td>
<td>27.0</td>
</tr>
<tr>
<td>Ozone</td>
<td>26.3</td>
<td>9.3</td>
<td>4.0</td>
</tr>
</tbody>
</table>

TABLE IV

D-VALUES (MIN) OF *BACILLUS SUBTILIS* SPORES AFTER PRETREATMENT WITH 6 PPM OZONE SOLUTION FOR 1 MIN AND SUBSEQUENTLY HEATING AT 85–95°C
Ozone has a maximum absorption for UV radiation at 253.7 nm. In a gaseous phase enriched with water vapor, photolysis of ozone involves the release of a molecule of oxygen and an atom of oxygen (McGrath and Norrish, 1960); the latter may react with water to produce hydroxyl radicals. Treatment of water by ozone and UV combination is achieved by placing a UV bulb in the ozone-contacting chamber (Graham, 2000). As water flows through the chamber, the UV bulb is turned on to initiate the decomposition of ozone molecules to hydroxyl radicals.

Kruithof and Kamp (1999) applied advanced oxidation processes in drinking waters spiked with pesticides or with clostridia spores. Ozone/peroxide and UV/peroxide combinations rapidly destroyed the pesticides tested; however, ozone treatment alone was not effective. Clostridia spores ($2.2 \times 10^4$ spores mL$^{-1}$) were resistant to ozone/peroxide and ozone/UV combination treatments, but, spore count decreased to below detection level with the UV/peroxide treatment.

Diaz and Law (1999) evaluated UV-ozonation system for the treatment of unscreened overflow poultry chiller water samples and obtained a more than 60% decrease in total plate count, coliforms and $E. \ coli$, and more than 80% of the light transmission of fresh water. Synergy between ozone and UV radiation accounted for $>0.8$ log unit decrease in total plate count. A synergistic bactericidal effect also was reported between ozone and UV radiation when poultry overflow chiller water, inoculated with nalidixic acid-resistant $S. \ typhimurium$, was treated with the combination for 4–8 min.

**F. OZONE AND PULSED ELECTRIC FIELD**

Unal et al. (2001) explored the potential enhancement of inactivation of *Lactobacillus leichmannii*, *E. coli* O157:H7 and *L. monocytogenes* by use of a pulsed electric field (PEF) when they are pretreated with ozone. The authors found a synergistic bactericidal effect. The *E. coli* count decreased by 3.6 log units and the *L. monocytogenes* count by 3.9 log units when these bacteria were treated sequentially with 0.75 mg ozone mL$^{-1}$ and 15 kV cm$^{-1}$. However, ozone at 0.75 mg L$^{-1}$ inactivated *E. coli* and *L. monocytogenes* by 1.8 and 3.0 log units, and PEF at 15 kV cm$^{-1}$ inactivated the microorganisms by 1.8 and 0.8 log units, respectively. The synergy was more apparent at mild than severe doses of ozone, and when the combination treatment was applied to *Lb. leichmannii* as opposed to *E. coli* or *L. monocytogenes*. Oshima et al. (1997) treated *E. coli* with combinations of PEF and ozone. They reported that simultaneous application of PEF and ozone synergistically inactivated *E. coli*. Their
data, however, show that ozone and PEF combinations had an additive rather than a synergistic effect.

VIII. ANALYTICAL METHODS

Ozone can be produced by electric discharge, photochemical, chemical, thermal, chemonuclear, and electrolytic methods (Horvath et al., 1985). The corona discharge method is commonly used to produce large amounts of ozone but the UV-based methods generate smaller yield and concentrations of the gas. The corona discharge produces ozone when a high-voltage alternating current is applied across a discharge gap in the presence of oxygen or air (Kim et al., 1999b).

There are physical, physicochemical, and chemical methods for the measurement of ozone. Most of the ozone analytical methods are modifications of chlorine residual methods, which are based on determining the total oxidation in solution. Physical methods measure direct absorption in the UV, visible or infrared region of the spectrum. Physicochemical methods are dependent upon reaction outputs such as heat or chemiluminescence. Chemical methods quantitate products released from the reaction between ozone and a chemical reagent such as potassium iodide.

Determination of residual ozone in aqueous solutions is quite difficult because of the rapid decomposition of ozone, volatility from solution, and reactivity with many organic and inorganic chemicals. The iodometric method has been widely used (Gordon and Grunwell, 1983). This method, however, measures ozone and other oxidizing species present in an ozone reaction solution. In addition, several factors, including pH, buffer composition, buffer concentration, iodide ion concentration, sampling techniques and reaction time, affect the accuracy of the iodometric method. Hence, measurement of residual ozone by the iodometric method is not recommended. The indigo method currently is widely used for determining residual ozone in water and waste water (APHA, 1998); it is relatively sensitive, precise and fast, with a detection limit of 0.005 mg L\(^{-1}\) ozone (Bader and Hoigné, 1981). Indigo has a relatively high molar absorptivity (~20 000 mol\(^{-1}\)cm\(^{-1}\)) and the dye absorbs light at 600–610 nm. Ozone reacts selectively with the carbon–carbon double bond of the sulfonated indigo molecule; therefore, ozone measurement by this method is not affected by the presence of hydrogen peroxide, organic peroxides, manganous ions and oxidized species in the aqueous medium. For gaseous ozone measurements, a UV spectrophotometric method, which is based on UV absorbance at 258 nm and a molar absorptivity of 2900 mol\(^{-1}\)cm\(^{-1}\), is most suitable.
In-line measurements of ozone are based on spectrophotometric, calorimetric or potentiometric methods. Several manufacturers produce instruments to monitor ozone concentration based on UV absorption. Calorimetric methods of ozone measurement depend upon decomposition of ozone in the presence of a catalyst. Residual ozone in water can also be measured by amperometric or potentiometric methods. The latter methods depend on the oxidation–reduction potential of ozone. In our laboratory, we compared ozone measurements using a commercial ozone analyzer (Ebara Jitsugyo Co., Ltd., Tokyo, Japan), the indigo-based chemical method (Hoigné and Bader, 1986) and a UV spectrophotometric continuous procedure (Kim and Yousef, 2000b). Results (Figure 8) show that ozone measurements by the commercial analyzer were consistently smaller, compared with those from the other methods (unpublished data). The length of the tube between the ozone reservoir and the analyzer seems to affect the discrepancy between the results. In addition, the length of the tube between the ozone reservoir and the UV spectrophotometer also cause variations in ozone concentration readings by this instrument. Since ozone is such a dynamic oxidizing agent, there should be a minimal time lag between collection of a sample and analysis. Consideration should also be given to the ozone demand of materials in contact with the sanitizer, e.g., the holding and handling apparatus.

![Figure 8](Image)

**FIG. 8.** Monitoring ozonation of water for 14 min using the indigo-based chemical method, a UV-spectrophotometric method, and a commercial ozone analyzer (Monitor).
IX. REGULATORY STATUS

Ozone use is permitted in many European and Asian countries. Ozone has been safely and effectively used in water treatment for several decades in European countries (Bryant et al., 1992). Industrial use of ozone in the United States was limited to the removal of metal ions, color, taste and odors in water (O’Donovan, 1965). In 1975, the US-FDA permitted the use of gaseous ozone up to 0.1 ppm in meat-aging coolers and in 1982, ozone was approved as a Generally Recognized As Safe (GRAS) substance for treatment of bottled water (USDA, 1982). According to the 1982 ruling, other uses of ozone (e.g. in food) require a food additive petition. Subsequently, the United States Department of Agriculture (USDA) permitted the use of ozone in poultry chiller water (USDA, 1984). The Electric Power Research Institute and Agriculture and Food Technology Alliance submitted a direct food additive petition to FDA so that ozone could be used in food processing without limitations (Graham, 2000). In response to the petition, the FDA amended the food additive regulations to provide for the safe use of ozone in gaseous and aqueous phases as an antimicrobial agent on food, including meat and poultry (Federal Register, 2001). This approval should boost a broad use of ozone in food processing.

X. LIMITATIONS, TOXICITY AND SAFETY

The reactivity of ozone and the potential deterioration in the quality of treated product may limit uses of this sanitizer in food processing. Sensory attributes may be altered, depending on the chemical composition of food, ozone dose and treatment conditions. Surface oxidation of food by ozone results in discoloration, undesirable odors and oxidative spoilage. Ozone decreased vitamins and amino acid contents and increased lipid oxidation and activity of some enzymes such as superoxide dismutase, ascorbate peroxidase, and glutathione reductase in lettuce leaves (Kang et al., 1999).

The acute and chronic effects of excessive exposure to ozone were investigated (Stockinger, 1965). Small concentrations of ozone gas in air (0.3 ppm) may cause discomfort to susceptible people. Scott and Lesher (1963) reported that as little as 0.02–0.04 mg L\(^{-1}\) can be detected by humans and 0.1 mg L\(^{-1}\) is objectionable to all normal humans because of irritation to the nose, throat and eyes. The respiratory tract is the primary site of ozone toxicity, where the gas may cause pulmonary congestion. Symptoms resulting from exposure to ozone include headaches and dryness of the throat, nose and eyes (Stockinger, 1965; Mustafa et al., 1980). Some researchers demonstrated that repeated exposures have progressively
lesser effects, suggesting that tolerance may develop (Nadel, 1979). Thorp (1950) indicated that with an hour exposure symptomatic, irritant, toxic and irreversible lethal effects can be induced by ozone concentrations of 2, 4, 15 and 95 ppm, respectively. Menzel (1984) postulated that ozone may generate toxic substance when tissue proteins, unsaturated fatty acids or other components of food are oxidized. However, immersion of shrimp meat in saline containing 5 mg L\(^{-1}\) ozone, for 120 min, did not generate mutagens in the product (Chen et al., 1992).

In a recent direct food additive petition, Graham (2000) argued that ozone can be used comparatively safely in industrial applications. According to the author, ozone is detected by human olfactory senses at concentrations as low as 0.01 ppm, and higher concentrations of the agent exert only temporary acute symptoms in humans. Since ozone has a high oxidation potential, disinfection can be accomplished with less concentration and shorter exposure time, compared to other oxidizing agents. Ozone is manufactured on-site at relatively low concentrations and pressures, therefore, an uncontrolled, widespread, and immediate release of large quantities of ozone is not possible. Graham (2000) also indicated that the relatively short half-life of ozone minimizes the persistence of the gas in the environment, and ozone breakdown product (diatomic oxygen) cause no harm.

When ozone is generated and used in food applications, precautions and personal safety always should be observed. Dissolving ozone in water is commonly accompanied by excess undissolved gas that remains entrapped in the solution (Hampson, 2000). Excess ozone should be degassed or separated from the water stream prior to delivery to equipment or the processing environment. Ozone detection and destruction systems and respirators are needed for the safety of workers in food processing facilities. Good manufacturing practice (GMP) and hazard analysis and critical control point (HACCP) systems are needed to control high ozone demand materials in food processing; this helps optimize ozone use in the processing facility. Workplace monitoring for ozone off-gas should be performed, and records should be maintained to ensure compliance with regulation.

Pryor and Rice (2000) discussed ozone exposure threshold limits. In the United States, current permissible exposure level-time weighted average (PEL-TWA) for ozone exposure in the work place environment is 0.1 ppm, as recommended by the American Conference of Governmental Industrial Hygienists (ACGIH, 1986) and adopted by the United States Occupational Safety and Health Administration (OSHA). Susceptible individuals can be exposed continually to this ozone concentration during a normal 8-h day/40-h working-week without adverse effects (CFR, 1997). The short-
term exposure limit is 0.3 ppm for an exposure less than 15 min and four times per day.

ACKNOWLEDGEMENTS

Funds for compiling this article were provided by the Center for Advanced Processing and Packaging Studies (Raleigh, NC; Columbus, OH; Davis, CA), and the US National Science Foundation.

REFERENCES

ACGIH 1986. “Threshold limit values for chemical substances in the work environment.” American Conference of Governmental Industrial Hygienists, Cincinnati, OH.


Garg, N., Churey, J. J., and Splittstoesser, D. F. 1990. Effect of processing conditions on the...
Hoigné, J., and Bader, H. 1975. Ozonation of water: role of hydroxyl radicals as oxidizing


Hwang, E. S. 1999. Post harvest treatment to reduce or remove ethylene bisdithiocarbamate (EBDC) fungicide residues from apples and apple products and elucidation of possible degradation by-products and pathways. Ph.D. Dissertation, Michigan State University.


Naitoh, S., Sawada, Y., and Yamaguchi, N. 1989. Studies on utilization of ozone in food


Rosenthal, H. 1974. Selected bibliography of ozone, its biological effects and technical applications. Fisheries research board of Canada technical report No. 456, Fisheries and Marine Service, Pacific Biological Station, Nanaimo, BC.


Zhang, S., and Farber, J. M. 1996. The effects of various disinfectants against *Listeria*

218 J.-G. KIM ET AL.
THE HIGH MOLECULAR WEIGHT SUBUNITS OF WHEAT GLUTENIN AND THEIR ROLE IN DETERMINING WHEAT PROCESSING PROPERTIES

PETER R. SHEWRY, NIGEL G. HALFORD AND ARTHUR S. TATHAM
IACR-Long Ashton Research Station
Department of Agricultural Sciences, University of Bristol
Long Ashton, Bristol BS41 9AF, UK

YVES POPINEAU
Institut National de la Recherche Agronomique
Centre de Recherches de Nantes
Laboratoire de Biochimie et de Technologie des Protéines
B.P. 71627, Rue de la Géraudière, Nantes 44316, Cedex 03, France

DOMENICO LAFIANDRA
Dipartimento di Agrobiologia ed Agrochimica
Università degli Studi della Tuscia
Via San Camillo de Lellis, Viterbo 01100, Italy

PETER S. BELTON
School of Chemical Sciences
University of East Anglia, Norwich NR4 7TJ, UK

I. Introduction
   A. Overview of Wheat Gluten Proteins
II. The HMW Subunits of Glutenin
   A. Genetics and Polymorphism of HMW Subunits
   B. Correlations between HMW Subunit Composition and Grain Processing Quality
III. The Sequences and Structures of HMW Subunits
   A. Amino Acid Sequences
   B. Size and Shape of HMW Subunits
   C. Structures of HMW Subunit Terminal Domains
   D. Structure of HMW Subunit Repetitive Domains
   E. Cross-link Formation between HMW Subunits
I. INTRODUCTION

The mature wheat grain contains about 9–15% protein, approximately half of which is storage proteins deposited in the starchy endosperm cells. These cells are separated from the outer layers (including aleurone) and embryo during milling to give white flour. When the white flour is mixed with water and kneaded to form dough the storage proteins form a continuous network, called gluten, which confers unique mechanical properties that underpin the utilization of wheat in many food systems. These properties are a combination of elasticity and viscosity, and the precise balance between these properties (dough strength) is important in determining the end use. In particular, relatively strong (i.e. highly elastic) doughs are required to make pan breads and weaker doughs to make flat breads, noodles, cakes and biscuits.

Wheat gluten can be readily prepared by washing dough with water, giving a cohesive mass comprising about 80% protein, 10% starch, 5%
lipid and 5% other components (minerals, fibre, etc.) on a dry weight basis. This has facilitated studies of the composition and biophysical properties of gluten but it should not be forgotten that these properties are also likely to be affected by interactions with other components in dough.

Because of their importance in wheat processing, the structures and properties of the wheat gluten proteins have been studied for many years, starting with the first description of the isolation of gluten by Beccari in 1745 (Beccari, 1745). Subsequent studies showed that gluten proteins could be separated into two fractions, which were either soluble or insoluble in alcohol (Taddei, 1819), and this division, with some modifications, has remained in use to the present day, with the gluten proteins that are readily soluble in alcohol–water mixtures (e.g. 60–70% ethanol) being called gliadins and those that are insoluble being called glutenins. These fractions are also important as they have functional significance, with the glutenins being mainly responsible for gluten and dough viscosty and elasticity and the gliadins for plasticity and extensibility (as discussed below). However, we now know that the two fractions contain proteins that are structurally related, with the differences in solubility resulting from their presence as monomers that interact by noncovalent forces (gliadins), or as high molecular mass polymers stabilized by inter-chain disulphide bonds. When present as reduced subunits the glutenin proteins are also soluble in alcohol–water mixtures and can therefore be defined together with the gliadins as prolamins. The ratio of gliadin to glutenin proteins in dough and gluten is generally about 1 : 1, although this ratio may vary with genotype and growth conditions with resulting effects on dough strength (Doekes and Wennekes, 1982; Graybosch et al., 1995; Vereijken et al., 2000; Johansson et al., 2001).

A. OVERVIEW OF WHEAT GLUTEN PROTEINS

The gliadins and glutenins are not single homogeneous proteins but complex mixtures that can be separated using various electrophoretic and other procedures. In fact, the true extent of the complexity has never been conclusively established as it is difficult to determine the correspondence of components separated by different procedures or whether apparently single components actually contain two or more closely related proteins. However, it is generally accepted that at least 50 different gluten proteins are present in hexaploid bread wheat, and that only partial separation can be achieved by any single procedure, even by two-dimensional systems such as that shown in Figure 1. A high level of complexity is supported by the analysis of gluten protein genes, although the existence of pseudogenes (i.e. nonexpressed genes) makes it difficult to establish precise gene copy numbers (see, for example, Anderson and Greene, 1997).
The gliadin proteins are traditionally separated into four groups, called (α, β-γ, and ω-gliadins, based on their mobilities when separated by electrophoresis at low pH (ω-gliadins being slowest) (Figure 2). We now know that α- and β-gliadins are closely related in sequence and structure and it is usual to refer to both as α-type gliadins, as opposed to the γ-type gliadins which are more distantly related. Furthermore, the α-type and γ-type gliadins are often classified together as sulphur-rich (S-rich) prolamins, while the ω-gliadins form a separate S-poor group.

The glutenin polymers can be separated on the basis of their size, as discussed below. However, in order to separate their component subunits it is necessary to first reduce the inter-chain disulphide bonds. Once this is done the subunits can be separated by electrophoresis in the presence of the detergent sodium dodecylsulphate (SDS) into two broad groups of subunits called high molecular weight (HMW) and low molecular weight (LMW) subunits, as shown in Figure 2.

The low molecular weight subunits can be further classified into three groups: the D-type subunits, which appear to be related to ω-gliadins; the C-type, which comprise components related to α-type and γ-type gliadins, and the B-type, which form a discrete group of S-rich prolamins. The
HMW subunits are not closely related to any other gluten proteins and form a distinct group called the HMW prolams. This classification is summarized in Figure 3.

![Diagram of wheat gluten proteins](image)

**FIG. 2.** The groups of gliadins and glutenin subunits separated by lactate-PAGE and SDS-PAGE, respectively. Taken from Shewry et al. (1999), with permission.

**FIG. 3.** The classification and nomenclature of wheat gluten proteins.
Although the HMW subunits were only identified and defined as a group about 20 years ago, they have since become the most widely and intensively studied group of gluten proteins. This is because of work started in the late 1970s which demonstrated correlations between HMW subunit composition and grain quality. However, before discussing these correlations in detail it is first necessary to discuss the polymorphism and genetic control of the HMW subunits.

II. THE HMW SUBUNITS OF GLUTENIN

A. GENETICS AND POLYMORPHISM OF HMW SUBUNITS

The use of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for the analysis of the subunits resulting from the reduction of glutenin polymers allowed the determination of their genetic control. Wheat genetic stocks, such as the nulli-tetrasomic and ditelocentric lines analysed by Bietz et al. (1975), gave the first clear evidence for the chromosomal location of glutenin subunit genes in the bread wheat cv. Chinese Spring. Subsequently, the introduction of the discontinuous SDS-PAGE system of Laemmli (1970) and two-dimensional electrophoretic separations have given more detailed information on the genetics of HMW glutenin subunits and the extent of their polymorphism in different bread wheat cultivars (Lawrence and Shepherd, 1980; Holt et al., 1981; Payne and Lawrence, 1983). In particular, it is now firmly established that genes controlling the synthesis of HMW subunits are located on the long arms of the homoeologous group 1 chromosomes of hexaploid bread wheat at loci designated Glu-A1, Glu-B1 and Glu-D1, with each Glu-1 locus containing two tightly linked genes encoding subunits of high and low $M_r$, which are termed x- and y-type, respectively. These gene loci have also been mapped but contrasting results have been obtained (Payne et al., 1981b; Singh and Shepherd, 1988). DNA sequencing of the genes corresponding to HMW glutenin subunits has revealed the structural characteristics of the corresponding subunits, including the presence of a large repetitive central domain that may provide a basis for major and rapid structural changes in the Glu-1 genes by duplication and/or deletion of large segments as a result of unequal crossing over (Shewry et al., 1989). These processes, together with the accumulation of small insertions, deletions or point mutations, have resulted in the existence of large numbers of allelic forms of subunits encoded by each locus, as detected by current electrophoretic techniques (Figure 4). Based on electrophoretic analyses of about 300 wheat varieties, Payne and
FIG. 4. SDS-PAGE of HMW subunits from a range of genotypes of wheat showing allelic variation in the mobilities of proteins encoded by the Glu-A1, Glu-B1 and Glu-D1 loci. The numbering is according to Payne and Lawrence (1983), with subsequent modifications by other workers.
Lawrence (1983) identified three alleles at the Glu-A1 locus, eleven at the Glu-B1 locus and five at the Glu-D1 locus, and proposed a numbering system to designate the different alleles. Subsequent analyses of cultivars from different countries and of wheat collections stored in gene banks has resulted in a continued increase in the number of alleles detected at each of the three loci (Figure 4).

The detection of genes at the Glu-D1 and Glu-A1 loci encoding rare subunits with unusual high molecular mass (see for example lanes 6, 13 and 14 of Figure 4) and their characterization by polymerase chain reaction (PCR) have provided further evidence for the role of the sequence encoding the repetitive central domain in the molecular evolution of glutenin subunit genes (D’Ovidio et al., 1994; Tahir et al., 1996). Despite the fact that bread wheat possesses six HMW subunit genes, the number of expressed subunits ranges from three to five because of gene silencing processes that have occurred during its evolutionary history. In particular, the y-type gene present at the Glu-A1 locus is always silent in cultivated wheat, while the x-type gene at the same locus and the y-type gene at the Glu-B1 locus are expressed only in some cultivars. In contrast, the y-type subunit encoded by the Glu-A1 locus is expressed in cultivated and wild diploid wheats (Triticum monococcum ssp. monococcum and ssp. boeoticum, T. urartu), in the wild tetraploid wheat T. turgidum ssp. dicoccoides (Waines and Payne 1987; Levy et al., 1988) and in cultivated and wild forms of tetraploid wheats with the genomic formula AAGG (Margiotta et al., 1998).

Studies of variation in HMW glutenin subunits present in old cultivars or landraces have also resulted in the identification of unusual allelic variants or mutant types characterized by the absence of subunits that are normally present in current bread wheat cultivars. For example, the absence of both HMW glutenin subunits encoded by chromosome 1D was reported by Bietz et al. (1975) in seeds of the landrace Nap Hal, while lines lacking either x- or y-type subunits encoded by the Glu-D1 or Glu-B1 loci have also been identified (Payne et al., 1984; Lafiandra et al., 1988). This type of material is being used for the development of genetic stocks suitable for elucidating the composition–functionality relationships, as discussed in a later section.

B. CORRELATIONS BETWEEN HMW SUBUNIT COMPOSITION AND GRAIN PROCESSING QUALITY

The first direct correlation between HMW subunit composition and grain processing quality was reported by Payne et al. (1979), who showed a correlation between the presence of HMW subunit 1Ax and quality
(measured using the indirect SDS sedimentation test) in the progeny of a cross between wheat cultivars of good (Maris Wigeon) and poor (Maris Ranger) breadmaking quality. Further studies showed a similar correlation with the subunit pair 1Dx5 + 1Dy10 (Payne et al., 1981a). Although Payne and coworkers used an indirect test for breadmaking quality, an association of subunits 1Dx5 + 1Dy10 and 1Ax2* with loaf volume was subsequently reported by Moonen et al. (1982, 1983), while Burnouf and Bouriquet (1980) demonstrated that subunits 1Dx5 and 1Bx7 were present in cultivars with good breadmaking quality and high gluten strength. The association of specific subunits or pairs of subunits with good or poor breadmaking quality (i.e. high or low gluten strength) has since been confirmed by many studies carried out using a wide range of germplasm and by workers in many countries (as reviewed by Payne, 1987 and Shewry et al., 1989, 1992), with general agreement on the following:

1. Subunits encoded by all three genomes (A, B, D) may be associated with quality.
2. The subunit pair 1Dx5 + 1Dy10 encoded by chromosome 1D is associated with the highest quality, whereas the allelic pairs 1Dx2 + 1Dy12, 1Dx3 + 1Dy12 and 1Dx5 + 1Dy12 are all associated with poor quality.
3. The presence of an x-type subunit encoded by chromosome 1A (1Ax1 or 1Ax2*) is superior to the null (i.e. silent) allele.
4. The subunit pair 1Bx17 + 1By18 is generally superior to other alleles encoded by chromosome 1B.

Furthermore, the combination of information from a wide range of studies has allowed “quality scores” to be assigned to individual subunits or subunit pairs (Payne et al., 1987b; Branlard et al., 1992), as shown in Table I.

### TABLE I

<table>
<thead>
<tr>
<th>Locus</th>
<th>Glu-A1</th>
<th>Glu-B1</th>
<th>Glu-D1</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>–</td>
<td>–</td>
<td>5 + 10</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>17 + 18</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>2*</td>
<td>7 + 8</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>7 + 9</td>
<td>2 + 12</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>–</td>
<td>3 + 12</td>
</tr>
<tr>
<td>1</td>
<td>null</td>
<td>7</td>
<td>4 + 12</td>
</tr>
<tr>
<td>1</td>
<td>–</td>
<td>6 + 8</td>
<td>–</td>
</tr>
</tbody>
</table>

Taken from Payne et al. (1987b) with permission.
Quantitative analyses have shown that the HMW subunits account for up to about 12% of the total grain proteins, corresponding to about 1–1.7% of the flour dry weight (Seilmeier et al., 1991; Halford et al., 1992; Nicolas, 1997). Nevertheless, they account for between about 45 and 70% of the variation in breadmaking performance within European wheats (Branlard and Dardevet, 1985, Payne et al., 1987b, 1988a). Consequently, the quality scores assigned to the HMW subunits can be exploited to select for breadmaking performance in breeding programmes.

III. THE SEQUENCES AND STRUCTURES OF HMW SUBUNITS

Although early studies resulted in the direct determination of the N-terminal amino acid sequences of a number of HMW subunits purified from wheat grain (Field et al., 1982; Shewry et al., 1984), the determination of full sequences resulted only from the isolation and sequencing of the corresponding genes. As a result we now know the sequences of nine HMW subunit proteins from bread wheat, including forms encoded by all three genomes (see Table II), and of homeologues from related cultivated (T. timopheevi) and wild (T. tauschii, Aegilops cylindrica) species (Mackie et al., 1996; Wan et al., 2002).

A. AMINO ACID SEQUENCES

The subunits which occur most widely in bread wheat comprise between 627 (1Dy10) and 827 (1Dx5) amino acid residues, with $M_r$ ranging from 67 476 to 88 128 (Table II). Furthermore, their amino acid sequences can be divided into three distinct parts, or domains, with the central domains consisting of highly repeated blocks of amino acids and ranging in length from 481 to 696 residues (Figure 5).

The availability of complete amino acid sequences of HMW subunit proteins from cultivated wheat and related wild species of grasses allows a detailed comparison of their repeat motifs to be made. Because there are no apparent differences between the sequences of proteins from modern bread wheat, ancient cultivated wheats (T. timopheevi) and wild related species (A. cylindrica, T. tauschii), data from these species are combined in Table III and in the following discussion.

A major difference between x-type and y-type subunits is that the former contain tripeptide, hexapeptide and nonapeptide motifs (Table III), whereas the y-type subunits contain only hexapeptides and nonapeptides (Table III). The tripeptides in x-type subunits always occur in tandem with hexapeptides, forming essentially a nine-residue repeat motif (see Figure 5).
TABLE II
SUMMARY OF THE TOTAL NUMBER OF RESIDUES (RES), NUMBER OF CYSTEINE RESIDUES (CYS) AND NUMBER OF TRI-, HEXA- AND NONAPEPTIDE REPEAT UNITS (TRI, HEXA, NONA, RESPECTIVELY) IN EIGHT HMW SUBUNITS OF GLUTENIN FROM BREAD WHEAT AND THEIR THREE STRUCTURAL DOMAINS

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Cultivar</th>
<th>$M_r$</th>
<th>Res</th>
<th>Cys</th>
<th>Res</th>
<th>Cys</th>
<th>Tri</th>
<th>Hexa</th>
<th>Nona&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Whole protein</td>
</tr>
<tr>
<td>1Ax1</td>
<td>Hope</td>
<td>87680</td>
<td>86</td>
<td>3</td>
<td>681</td>
<td>0</td>
<td>15</td>
<td>65</td>
<td>23</td>
</tr>
<tr>
<td>1Ax2*</td>
<td>Cheyenne</td>
<td>86309</td>
<td>86</td>
<td>3</td>
<td>666</td>
<td>0</td>
<td>16</td>
<td>67</td>
<td>23</td>
</tr>
<tr>
<td>1Bx7</td>
<td>Cheyenne</td>
<td>82865</td>
<td>81</td>
<td>3</td>
<td>647</td>
<td>0</td>
<td>4</td>
<td>66</td>
<td>25</td>
</tr>
<tr>
<td>1Bx17</td>
<td>L88-69</td>
<td>80750</td>
<td>81</td>
<td>3</td>
<td>611</td>
<td>0</td>
<td>4</td>
<td>64</td>
<td>23</td>
</tr>
<tr>
<td>1By9</td>
<td>Cheyenne</td>
<td>73518</td>
<td>104</td>
<td>5</td>
<td>538</td>
<td>1</td>
<td>0</td>
<td>56</td>
<td>22</td>
</tr>
<tr>
<td>1Dx2</td>
<td>Yamhill</td>
<td>87000</td>
<td>88</td>
<td>3</td>
<td>687</td>
<td>0</td>
<td>20</td>
<td>73</td>
<td>21</td>
</tr>
<tr>
<td>1Dx5</td>
<td>Cheyenne</td>
<td>88137</td>
<td>89</td>
<td>3</td>
<td>696</td>
<td>1</td>
<td>23</td>
<td>73</td>
<td>21</td>
</tr>
<tr>
<td>1Dy10</td>
<td>Cheyenne</td>
<td>67495</td>
<td>104</td>
<td>5</td>
<td>481</td>
<td>1</td>
<td>0</td>
<td>47</td>
<td>21</td>
</tr>
<tr>
<td>1Dy12</td>
<td>Chinese Spring</td>
<td>68696</td>
<td>104</td>
<td>5</td>
<td>493</td>
<td>1</td>
<td>0</td>
<td>49</td>
<td>21</td>
</tr>
</tbody>
</table>

<sup>a</sup>Includes two long (11/12 residue) degenerate repeats.

Based on sequences reported in Halford et al. (1987, 1992); Anderson and Greene (1989); Anderson et al. (1989); Thompson et al. (1985); Sugiyama et al. (1985); Reddy and Appels (1993).
Similarly, in both types of subunit the hexapeptides occur either in tandem arrays or interspersed with nonapeptides, the latter forming a 15-residue repeat (Figure 6). Unlike the hexapeptides, the tripeptides and nonapeptides never occur in tandem arrays.

The hexapeptides have the same consensus motif in x-type and y-type subunits (Pro.Gly.Gln.Gly.Gln.Gln.), but subtle differences are apparent in the frequencies of substitutions at different positions. Notably, the replacement of Pro with Ser at position 1 is much more common in x-type subunits, as is the replacement of Gln with Pro at position 6. However, the latter only occurs in hexapeptides within a 15-residue (i.e. 6 + 9) motif, not in tandemly arranged hexapeptides. Most, but not all, of the 15 amino acid repeats in x-type subunits have Pro in this position.

Greater differences are present between the nonapeptides in the x-type and y-type subunits. Replacement of Tyr with His at position 2 and of Thr with Ala at position 5 are both common in y-type subunits but rare or absent in x-type. These two substitutions also usually occur together, resulting in two consensus sequences for y-type nonapeptides: Gly.Tyr.Tyr.Pro.Thr.Ser.Leu.Gln.Gln. and Gly.His.Tyr.Pro.Ala.Ser.Leu.Gln.Gln. The consensus motif of the x-type subunits also differs in having Pro in place of Leu at position 7.

The data in Table III also show clear differences in the frequency of amino acid substitutions at different position in the motifs. In particular, Gln is highly conserved wherever it occurs (positions 3, 5 and 6 of the nonapeptide and 2 and 3 of the tripeptide), with the exception of the substitution with Pro at position 6 of hexapeptides present in 15-residue repeats of x-type subunits, as discussed above. These conserved glutamine residues can, therefore, be regarded as forming a glutamine backbone to the repetitive domains.
### TABLE III

FREQUENCY OF OCCURRENCE OF DIFFERENT AMINO ACID RESIDUES IN EACH POSITION OF: (A) THE 347 HEXAPEPTIDE, 103 NONAPEPTIDE AND 81 TRIPEPTIDE REPEAT MOTIFS OF x-TYPE HMW SUBUNITS 1Ax1, 1Bx7 AND 1Dx5 (*T. aestivum*); 1Ax (*T. timopheevi*); 1Dx (*Ae. cylindrica*). (B) THE 339 HEXAPEPTIDE AND 123 NONAPEPTIDE REPEAT MOTIFS OF y-TYPE HMW SUBUNITS 1Ay (NOT EXPRESSED), 1By9 AND 1Dy10 (*T. aestivum*); 1Ay (*T. timopheevi*); 1Cy AND 1Dy (*Ae. cylindrica*); 1Dy (*T. tauschii*).

<table>
<thead>
<tr>
<th>Hexapeptides (%)</th>
<th>Tripeptides (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pro 62</td>
<td>Gly 89</td>
</tr>
<tr>
<td>Gly 84</td>
<td>Gln 99</td>
</tr>
<tr>
<td>Gln 99</td>
<td>Gly 75</td>
</tr>
<tr>
<td>Gly 75</td>
<td>Gln 94</td>
</tr>
<tr>
<td>Gln 94</td>
<td>Gln 80</td>
</tr>
<tr>
<td>Gly 89</td>
<td>Arg 1</td>
</tr>
<tr>
<td>Gly 99</td>
<td>Arg 1</td>
</tr>
<tr>
<td>Ser 26</td>
<td>Ala 7</td>
</tr>
<tr>
<td>Ala 7</td>
<td>1 Other</td>
</tr>
<tr>
<td>1 Other</td>
<td>Trp 9</td>
</tr>
<tr>
<td>Trp 9</td>
<td>Leu 3</td>
</tr>
<tr>
<td>Leu 3</td>
<td>Pro 15</td>
</tr>
<tr>
<td>Pro 15</td>
<td>Gly 89</td>
</tr>
<tr>
<td>1</td>
<td>Gln 99</td>
</tr>
<tr>
<td>1</td>
<td>Gln 99</td>
</tr>
<tr>
<td>Ile 1</td>
<td>Arg 3</td>
</tr>
<tr>
<td>Arg 3</td>
<td>Glu 4</td>
</tr>
<tr>
<td>Glu 4</td>
<td>Arg 1</td>
</tr>
<tr>
<td>Arg 1</td>
<td>Arg 2</td>
</tr>
<tr>
<td>Other 2</td>
<td>Thr 2</td>
</tr>
<tr>
<td>Thr 2</td>
<td>Arg 2</td>
</tr>
<tr>
<td>Arg 2</td>
<td>Leu 1</td>
</tr>
<tr>
<td>Leu 1</td>
<td>1 Ala 1</td>
</tr>
<tr>
<td>Ala 1</td>
<td>Other 2</td>
</tr>
<tr>
<td>Other 2</td>
<td></td>
</tr>
<tr>
<td><strong>Nonapeptides (%)</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gly 84</td>
<td>Tyr 98</td>
</tr>
<tr>
<td>Tyr 98</td>
<td>Tyr 97</td>
</tr>
<tr>
<td>Tyr 97</td>
<td>Pro 90</td>
</tr>
<tr>
<td>Pro 90</td>
<td>Thr 96</td>
</tr>
<tr>
<td>Thr 96</td>
<td>Ser 100</td>
</tr>
<tr>
<td>Ser 100</td>
<td>Gly 88</td>
</tr>
<tr>
<td>Gly 88</td>
<td>Gln 94</td>
</tr>
<tr>
<td>Arg 6</td>
<td>His 2</td>
</tr>
<tr>
<td>His 2</td>
<td>Asp 2</td>
</tr>
<tr>
<td>Asp 2</td>
<td>Leu 8</td>
</tr>
<tr>
<td>Leu 8</td>
<td>Ile 4</td>
</tr>
<tr>
<td>Ile 4</td>
<td>Ser 2</td>
</tr>
<tr>
<td>Ser 2</td>
<td></td>
</tr>
<tr>
<td>Trp 3</td>
<td>Phe 1</td>
</tr>
<tr>
<td>Phe 1</td>
<td></td>
</tr>
<tr>
<td>Trp 3</td>
<td></td>
</tr>
<tr>
<td>Val 2</td>
<td></td>
</tr>
<tr>
<td>Val 2</td>
<td>Ala 2</td>
</tr>
<tr>
<td>Ala 2</td>
<td>Arg 1</td>
</tr>
<tr>
<td>Ala 1</td>
<td></td>
</tr>
<tr>
<td>Lys 1</td>
<td></td>
</tr>
<tr>
<td>Lys 1</td>
<td></td>
</tr>
<tr>
<td><strong>Hexapeptides (%)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>B</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pro 65</td>
<td>Gly 92</td>
</tr>
<tr>
<td>Gly 96</td>
<td>Gln 96</td>
</tr>
<tr>
<td>Gln 96</td>
<td>Gly 76</td>
</tr>
<tr>
<td>Gly 76</td>
<td>Gln 94</td>
</tr>
<tr>
<td>Gln 94</td>
<td>Gln 94</td>
</tr>
<tr>
<td>Arg 12</td>
<td>Glu 6</td>
</tr>
<tr>
<td>Glu 6</td>
<td>Lys 4</td>
</tr>
<tr>
<td>Lys 4</td>
<td></td>
</tr>
<tr>
<td>Leu 10</td>
<td>Trp 7</td>
</tr>
<tr>
<td>Trp 7</td>
<td>Lys 2</td>
</tr>
<tr>
<td>Lys 2</td>
<td>His 2</td>
</tr>
<tr>
<td>His 2</td>
<td></td>
</tr>
<tr>
<td>Ile 7</td>
<td>Other 1</td>
</tr>
<tr>
<td>Other 1</td>
<td>Arg 4</td>
</tr>
<tr>
<td>Arg 4</td>
<td>Other 1</td>
</tr>
<tr>
<td>Other 1</td>
<td></td>
</tr>
<tr>
<td>Thr 4</td>
<td>Ala 2</td>
</tr>
<tr>
<td>Ala 2</td>
<td></td>
</tr>
<tr>
<td>Gly 1</td>
<td>Val 2</td>
</tr>
<tr>
<td>Val 2</td>
<td></td>
</tr>
<tr>
<td>Other 1</td>
<td>Lys 1</td>
</tr>
<tr>
<td>Lys 1</td>
<td></td>
</tr>
<tr>
<td><strong>Nonapeptides (%)</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gly 96</td>
<td>Tyr 54</td>
</tr>
<tr>
<td>Tyr 54</td>
<td>Tyr 85</td>
</tr>
<tr>
<td>Tyr 85</td>
<td>Pro 91</td>
</tr>
<tr>
<td>Pro 91</td>
<td>Thr 60</td>
</tr>
<tr>
<td>Thr 60</td>
<td>Ser 97</td>
</tr>
<tr>
<td>Ser 97</td>
<td>Leu 54</td>
</tr>
<tr>
<td>Leu 54</td>
<td>Gln 97</td>
</tr>
<tr>
<td>Gln 97</td>
<td>Gln 90</td>
</tr>
<tr>
<td>Trp 2</td>
<td>His 41</td>
</tr>
<tr>
<td>His 41</td>
<td>Cys 4</td>
</tr>
<tr>
<td>Cys 4</td>
<td>Leu 5</td>
</tr>
<tr>
<td>Leu 5</td>
<td>Ala 37</td>
</tr>
<tr>
<td>Ala 37</td>
<td>Tyr 2</td>
</tr>
<tr>
<td>Tyr 2</td>
<td></td>
</tr>
<tr>
<td>Arg 1</td>
<td>Gln 5</td>
</tr>
<tr>
<td>Gln 5</td>
<td>Asp 2</td>
</tr>
<tr>
<td>Asp 2</td>
<td>Arg 2</td>
</tr>
<tr>
<td>Arg 2</td>
<td>Ser 2</td>
</tr>
<tr>
<td>Ser 2</td>
<td>Phe 1</td>
</tr>
<tr>
<td>Phe 1</td>
<td></td>
</tr>
<tr>
<td>Val 4</td>
<td></td>
</tr>
<tr>
<td>Stop 1</td>
<td></td>
</tr>
<tr>
<td>Ile 2</td>
<td>Thr 1</td>
</tr>
<tr>
<td>Thr 1</td>
<td></td>
</tr>
<tr>
<td>Ile 2</td>
<td></td>
</tr>
<tr>
<td>Gly 2</td>
<td></td>
</tr>
<tr>
<td>Phe 2</td>
<td></td>
</tr>
<tr>
<td>Arg 1</td>
<td></td>
</tr>
<tr>
<td>Asn 1</td>
<td></td>
</tr>
<tr>
<td>Glu 1</td>
<td></td>
</tr>
</tbody>
</table>

Percentages may not add up to 100 because of rounding. Residues present at less than 1% are either included as ‘Other’ if together they add up to 1% or are not shown.
FIG. 6. Amino acid sequences of the repetitive domains of typical x-type (1Dx5) and y-type (1Dy10) HMW subunits arranged to show their repeat unit structures.
The x-type subunits are also highly conserved at positions 2, 3, 5, 6 and 9 of the nonapeptide, with the y-type subunits being more variable at these positions. Similarly, Ser is always present at position 6 of the x-type nonapeptides and is highly conserved (97%) in the y-type motifs.

However, comparison of the properties of the amino acids that occur at specific positions provides no evidence for conservative substitutions (i.e. replacement with an amino acid with similar properties). Instead, the most common replacements can be accounted for by single base mutations in the DNA codons, with substitutions requiring two mutations occurring more rarely. For example, 55% of the x-type hexapaptides have Pro (codon CCA) at position 1, with 12% containing Leu (CTA), 30% Ser (TCA) but only 3% Ile (ATA). Nevertheless it is possible that at least some of the differences in the degree of conservation within motifs relate to the role of individual residues in determining protein structure.

A number of studies have been carried out to determine the structures of the HMW subunits and their domains. However, it must be borne in mind that most of these have been carried out on proteins or peptides in the solution state rather than as hydrated solid, which is the “natural” state in protein bodies in the developing grain and in gluten and dough.

B. SIZE AND SHAPE OF HMW SUBUNITS

Data from a range of studies indicate that the HMW subunits have an extended rod-like structure, both in solution and the hydrated solid state (Table IV). Field et al. (1987) used intrinsic viscosity measurements to calculate the dimensions of subunit 1Bx20 purified from pasta wheat, showing overall dimensions ranging from $49 \times 1.8$ nm in 50% (v/v) aqueous propan-1-ol to $62 \times 1.5$ nm in trifluoroethanol. Small angle X-ray scattering (SAXS) has shown similar lengths but different diameters, of $56.7 \times 8.0$ and $78.6 \times 6.3$ nm for undefined subunits in 50% (v/v) aqueous propan-1-ol and 0.1 m acetic acid, respectively (Matsushima et al., 1992), and of $69 \times 6.4$ nm for subunit 1Bx20 in 50% (v/v) aqueous propan-1-ol (Thomson et al., 1999).

Scanning tunnelling microscopy (STM) of the hydrated solid protein showed aligned rods of diameter about 1.8 nm (Miles et al., 1991). It can therefore be suggested that the diameters obtained by SAXS represent side-to-side aggregates rather than individual proteins. Atomic force microscopy (AFM) of subunit 1Dx5 deposited onto mica or graphite substrate has indeed shown such filaments, with a diameter of about 20 nm (Figure 7). An $M_r$ 58 000 peptide derived from the central repetitive domain of subunit 1Dx5 also formed filamentous structures with diameters about 20 nm, with connection points every 45–50 nm (Humphris et al., 2000).
C. STRUCTURES OF HMW SUBUNIT TERMINAL DOMAINS

The N- and C-terminal domains of the HMW subunits have proved to be difficult to study in isolation from the central repetitive domain. Early structure predictions indicated that these domains were essentially globular in structure, being rich in $\alpha$-helix (Tatham et al., 1984, 1985). More recently, van Dijk et al. (1998) have predicted that residues 1–33 of subunit 1Dx5 (and the corresponding regions of subunits 1Ax1, 1Ax2* and 1Bx7) form $\alpha$-helices while residues 40–48, 52–59, 64–67 and 75–84 form $\beta$-sheet.

This is supported by more detailed prediction and modelling studies reported by Köhler et al. (1997). They proposed that residues 5–32 of subunit 1Dx5 form a continuous $\alpha$-helix, as shown in Figure 8. In contrast, several short sections of $\alpha$-helix are predicted for subunit 1Bx7 (residues 6–13, 16–20, 24–26), with an inverse $\beta$-turn between residues 14 and 16 allowing the formation of an intra-chain disulphide bond between the cysteine residues at positions 10 and 17 (Figure 8) (see below for a discussion of disulphide bond formation).

It has not so far proved possible to isolate a peptide corresponding to the N-terminal domain of an authentic HMW subunit, but van Dijk et al. (1998) have reported the expression in *Escherichia coli* and
FIG. 7. Atomic force microscopy of reduced and alkylated subunit 1Dx5 deposited from 0.05 M acetic acid on a highly orientated pyrolytic graphite (HOPG) substrate shows network formation. Taken from Humphris et al. (2000), with permission.
characterization of a peptide corresponding to the N-terminal domain of subunit 1Dx5. The peptide was insoluble in water except in the presence of SDS and circular dichroism spectroscopy (in 0.1% SDS) indicated the presence of 26% $\alpha$-helix and 33% $\beta$-sheet at pH 8.17 and 35% $\alpha$-helix and 36% $\beta$-sheet at pH 3.59. The authors concluded that the insolubility of the N-terminal domain was responsible for the solubility properties of the whole subunits. However, the expressed peptide differed from the native N-terminal domain in the presence of a 21-residue signal sequence and it is possible that this hydrophobic sequence may have affected the structure and properties of the peptide, including the solubility.

Tatham et al. (1984) also predicted that the short (42-residue) C-terminal domains of the HMW subunits were $\alpha$-helical in structure. Bekkers et al. (1996) subsequently synthesized a peptide corresponding to the C-terminal domain of subunit 1Dx5 and showed that it was readily soluble in aqueous buffers over a wide pH range and adopted a random coil structure when dissolved in water. Nuclear magnetic resonance (NMR) spectroscopy in the “structure-inducing” solvent 40% (v/v) aqueous trifluoroethanol allowed a low-resolution structure to be determined which was “molten globule”-like, with $\alpha$-helical regions formed by residues 5–20 and 26–32. It is not possible to conclude whether this structure is related to that adopted in the hydrated solid state (i.e. as in gluten) and it is also possible that the
structure of this domain, and of the N-terminal and central domains, is affected by interactions with other domains of the subunit or with other gluten proteins.

D. STRUCTURE OF HMW SUBUNIT REPETITIVE DOMAINS

The repetitive domains form the largest part of the HMW subunits, ranging from 481 to 696 residues. Initial studies using secondary structure prediction (Tatham et al., 1984, 1985), circular dichroism (CD) and infrared (IR) spectroscopies of the whole protein (Tatham et al., 1985) and synthetic peptides based on the repeat motifs (Tatham et al., 1990) indicated β-turns as the dominant structural feature. β-Turns were predicted over residues QPGQ, QQGY, YPTS and SPQQ, and it was proposed that regular β-turn predictions led to the formation of a spiral structure, similar to the β-spiral described for a synthetic polypentapeptide based on the repeat motif present in the connective tissue protein elastin (Tatham et al., 1985). Van Dijk et al. (1996a) used 2D-NMR, CD and IR of synthetic cyclic peptides to confirm the presence of β-turns: a type II β-turn at QPGQ, type I β-turns at YPTS and SPQQ and a type I or II β-turn at QQGY. They also reported cis/trans proline isomerism, with 50% of proline residues in the cis conformation in YPTS, and the other proline residues being more than 90% trans-conformation. Conversion of the cis form in YPTS to the trans-conformation destabilized the type I turn, but increased the stability of the β-turns at SPQQ and QQGY. Van Dijk et al. (1996b) also studied the solution structures of HMW subunits 1Bx6 and 1Bx7 and of an \( M_r 16802 \) heterologously expressed peptide from the central repetitive domain of subunit 1Dx5. Using CD and IR, they concluded that the structure was compatible with β-turns stabilized by hydrogen bonds both within and between turns. More recently, variable temperature CD studies of an \( M_r 58000 \) peptide derived from the central repetitive domain of subunit 1Dx5 indicated the presence of β-turn structures in equilibrium with a poly-L-proline II-like structure (an extended hydrated structure), with supporting evidence for this coming from IR studies (Gilbert et al., 2000).

Matsushima et al. (1990) attempted to model the nonapeptide repeat motif of an x-type subunit (GYYPTSPQQ), but concluded that as the repeat contained two proline residues, there were many degrees of freedom and hence many models could be constructed. They modelled structures with two or three β-turns per repeat and calculated diameters of 1.4–1.6 nm, but reported no detailed structures. Kasarda and coworkers have developed models based on hexapeptide and nonapeptide repeats and a hexanonapeptide (15-mer) repeat, fitted to the hexapeptide template (Kasarda, 1994; Kasarda et al., 1994). Using energy minimization and molecular
dynamics they reported that when type II $\beta$-turns were used a distorted spiral with a flat ribbon shape resulted, but that this was unstable and other turn types did not improve the stability. When inverse $\gamma$-turns (a three-residue turn) were modelled a more stable spiral structure resulted, with a diameter of about 2.4 nm and a pitch of about 1 nm. The stability of the spiral arose from hydrogen bonding of the glutamine side chain amide groups to the backbone amide groups and to other glutamine side chains. However, the model did not include interactions with water, which might break up some or all of the hydrogen bonding that was proposed (Kasarda et al., 1994).

Parchment et al. (2001) used structure prediction and molecular dynamics to generate three alternative spiral structures based largely on $\beta$-turns with diameters of about 2 nm. The $\beta$-turns were placed over residues identified by prediction and spectroscopic studies and spiral structures were generated in the presence of a water shell (Figure 9). The simplest model placed a type II $\beta$-turn at QPGQ and turns were forced at YPTS, SPQQ and QGQQ in the repeat motif, resulting in a spiral with a diameter of 1.7 nm and a pitch of 1.3 nm (Figure 9). In the second model type II $\beta$-turns were located at QPGQ and QGYY and type I/III $\beta$-turns at SPQQ and YPTS, resulting in a spiral structure with a diameter of about 2 nm and
pitch of 1.6 nm (Figure 9). The third model did not use standard $\beta$-turn types, but the positions of $\beta$-turns were based on distance criteria. These were located at QPGQ, YPTS and SPQQ, with the sequence GQGQQGY forming a $\beta$-sheet structure. The resulting spiral was flattened, with a cross-section of about $1.7 \times 2.5$ nm (Figure 9). Calculated dimensions for the models are shown in Table IV.

Arkin et al. (2000) used multicanonical simulation to model the structures of five common tetrapeptide sequences (QPGQ, QSGQ, YPTS, SPQQ and QPGY) in the central repetitive domains. They found that QOGQ and QPGY had the highest probabilities for $\beta$-turns, with lower probabilities for the others. They also considered the probabilities of $\gamma$-turn formation and found probabilities in all five tetrapeptides. They concluded that these may make a contribution to the overall structures of the central repetitive domains. Arkin et al. (2001) also modelled two hexapeptide repeats, PGQGQQ and SGQGQQ; these agreed with about 40% of the total occurrence of $\beta$-turns predicted by Tatham et al. (1985), but also concluded $\gamma$-turns may contribute to the proposed spiral structure.

The studies discussed above were either carried out on the HMW subunits in dilute solution, or were modelled in vacuo or hydrated in a water shell. However, the environment of the HMW subunits in dough and gluten is as a hydrated solid protein mass. Belton et al. (1995) used Fourier transform infrared (FT-IR) and NMR spectroscopy to study the hydration behaviour of subunits and found that the proportions of $\beta$-turns and $\beta$-sheet varied in relation to the water content. Thus, the content of intermolecular $\beta$-sheet structure increased when the protein was hydrated from the dry solid. Gilbert et al. (2000) also expressed an $M_r$ 58 000 peptide from the central repetitive domain of subunit 1Dx5 in E. coli and studied its behaviour during hydration using FT-IR. They reported that $\beta$-turn rich structures formed in the dry and hydrated solid states, with significant contents of intermolecular $\beta$-sheet structure. In comparison with intact subunits, the $M_r$ 58 000 peptide showed a lower propensity to form $\beta$-sheet structure, indicating that the N- and C-terminal domains may play a role in assembling the molecules to allow $\beta$-sheet formation to occur in the central repetitive domains. NMR studies indicate that the central repetitive domain is flexible, with increasing hydration resulting in increased flexibility (Belton et al., 1995; Alberti et al., 2001). However, two populations of glutamine residues were identified: one in a mobile environment that was tentatively identified with $\beta$-turn conformations, and a second population that was more hindered, possibly by hydrogen bonding, in protein segments containing glycine residues and possibly adopting a $\beta$-sheet conformation (Alberti et al., 2001).
E. CROSS-LINK FORMATION BETWEEN HMW SUBUNITS

The HMW subunits are only present in oligomers or polymers that are stabilized by inter-chain disulphide bonds. However, little is known about their organization within these polymers.

Partial reduction of gluten with reducing agents followed by stabilization of the free cysteine sulphhydryl groups with cystamine diHCl has been shown to result in the release of HMW subunit dimers (Lawrence and Payne, 1983; Werner et al., 1992). These include a preponderance of x–y dimers, and in particular dimers involving 1Dy with 1Ax, 1Bx or 1Dx subunits. These studies indicate that x–y HMW subunit dimers may act as “building blocks” in glutenin polymers.

Evidence for head-to-tail bonds between HMW subunits was reported by Tao et al. (1992). They prepared a glutenin-enriched fraction from flour and digested this with the endoproteinase LysC. Fractionation of the digest led to the isolation of two peptides containing intact disulphide bonds, both of which consisted of the C-terminal part of subunit 1Bx17 linked to the N-terminal domain of subunit 1Dy10. However, the precise cysteine residue involved in subunit 1Dy10 could not be identified.

The isolation of x–y dimers and the identification of head-to-tail disulphide bonds are both consistent with the suggestion by Graveland et al. (1985) that alternating x-type and y-type subunits form the “backbone” structure of the glutenin polymers.

Further disulphide bonds involving HMW subunits have been reported by Köhler and coworkers (Köhler et al., 1991, 1993, 1994; Keck et al., 1995). These include one intra-chain bond within the N-terminal domain of subunit 1Bx7 (involving Cys 10 and Cys 17) (Figure 8) and one cross-link between a cysteine residue within the repetitive domain of subunit 1By9 (Cys 564) or 1Dy10 (Cys 507) and a low molecular weight subunit of glutenin. This could lead to the presence of LMW subunits as “branches” on the HMW subunit backbone, as proposed by Graveland et al. (1985).

![Diagram](attachment:figure10.png)

FIG. 11. Hypothetical structure for wheat glutenin polymers, based on mapped disulphide bonds (Figure 10). Not all cysteine residues are included. The additional cysteine residue present in the repetitive domain of subunit 1Dx5 is proposed to link to another HMW subunit, based on the effect on gluten rheology of transformation with additional gene copies.
In addition, two disulphide bonds were identified between the adjacent cysteine residues (Cys 44, Cys 45) in the N-terminal domains of two y-type subunits (1By9 and/or 1By10), linking the two subunits in parallel. Since hetero- or homodimers comprising two y-type subunits were not detected by Werner et al. (1992) or Lawrence and Payne (1983), it can be concluded that such cross-links between y-type subunits are either readily reduced or occur at low frequency.

Based on these studies, our current knowledge of the disulphide bonds formed by HMW subunits is summarized in Figure 10. This information can be used to propose a wider structural model for wheat glutenin polymers, based on x–y subunit dimers with branches to LMW subunits via y-type subunits (Figure 11). A more detailed discussion of the disulphide bond structure of gluten proteins is provided by Shewry and Tatham (1997).

Although disulphide bonds are classically considered to be the only type of covalent cross-links in wheat glutenin polymers, Tilley et al. (2001) have recently reported that a novel type of cross-link may occur. These are tyrosine cross-links formed specifically between the Tyr.Tyr sequences present in the HMW subunit repeat motifs. Analysis of synthetic peptides (Tyr.Tyr.; Tyr.Tyr.Pro.Thr.Ser. and Gln.Gln.Gly.Tyr.Tyr.Pro.Thr.Ser.) demonstrated that the formation of such cross-links was enhanced by KBrO$_3$ which acts as an “improver” in breadmaking. The authors suggest that tyrosine bonds formed during mixing and baking contribute to the structure of the gluten network.

IV. EXPERIMENTAL EVIDENCE FOR THE ROLE OF HMW SUBUNITS IN DOUGH MIXING AND GLUTEN VISCOELASTICITY

Experimental evidence for the role of the HMW subunits in determining the viscoelastic properties of doughs comes from a range of studies in which gluten fractions, individual proteins and peptides have been studied in reconstituted systems.

A. GLUTEN FRACTIONATION AND RECONSTITUTION

A valuable approach to elucidate the role played by the different protein components in gluten and dough functionality is to fractionate and reconstitute gluten or flour and to observe the effects of adding or omitting components on the technological or rheological properties.

The earliest work used this approach to investigate the role of gliadin- and glutenin-rich fractions. In this way Finney and coworkers (for a synthesis, see Finney, 1985) showed that glutenin was involved in the mixing requirement
and that glutenins extracted from good or poor quality wheats had different properties. Further fractionation of glutenin into acid-soluble and acid-insoluble fractions, corresponding broadly to components with different polymer sizes and/or aggregation behaviour, showed a positive relationship between the acid-insoluble (or high molecular mass) glutenin aggregates and dough mixing strength (Orth and Bushuk, 1972; Huebner and Wall, 1976; Hamada et al., 1982). More recently, it has been shown that increasing the glutenin to gliadin ratio (while maintaining a constant total protein content) increased the mixing time and the overmixing tolerance of dough. Extensibility was reduced by the addition of glutenin whereas resistance to extension was increased (Uthayakumar et al., 1999), corresponding to higher rupture viscosity and a lower rupture strain when dough was submitted to uniaxial elongation. In contrast, gliadin contributed to increased extensibility of dough by lowering the viscosity and enhancing the rupture strain (Uthayakumar et al., 2000a).

Rheological studies have also been carried out on isolated gliadin and glutenin fractions separated by the Osborne procedure. Gliadins were described as an elasto-viscous liquid whereas glutenins behaved like a viscoelastic solid (Khatkar et al., 1995). The addition of protein fractions to gluten and gluten reconstitution with varied ratios of gliadins to glutenins showed that the storage (related to elasticity) and the loss (related to viscosity) moduli ($G'$ and $G''$, respectively) of gluten were positively related to its content of glutenin and that increasing the glutenin content increased gluten elasticity (Khatkar et al., 1995; Janssen et al., 1996).

However, the results of this approach could be invalidated if the extraction procedure modified protein functionality (MacRitchie, 1985; Skerritt et al., 1996) and it was considered that the extraction of gliadin with alcohol/water in the classical Osborne procedure could irreversibly change the aggregation of the glutenin fraction (Hoseney et al., 1969). MacRitchie (1987) therefore proposed a new method for the sequential extraction of gluten proteins in dilute hydrochloric acid, a procedure which was considered to preserve their original functionality. The ten fractions obtained in this way contain mixtures of gliadin and glutenin in varying proportions, with the size distribution of the glutenin polymers also varying between the fractions (Lundh and MacRitchie, 1989). The effects of these fractions on the mixing properties and the storage modulus ($G'$) of the dough, when added to a control flour, were related to their contents of glutenin polymers and to their aggregation state (MacRitchie, 1987; Eliasson and Lundh, 1989). The same fractionation procedure was also applied to gluten extracted in the laboratory. The fractions were analysed by size-exclusion high-performance liquid chromatography (SE-HPLC) under dissociating conditions (Cornec et al., 1994), showing that the most readily extracted material...
FIG. 12. Viscoelastic properties of gluten subfractions differing in their content and size distribution of glutenin polymers. The content of glutenin and the proportion of the largest glutenin polymers increase from fractions F3 to F8. Dynamic rheological assays in shear were performed at 20°C on gluten fractions fully hydrated with water using a Carri-Med CSL 100 constant stress rheometer (cone-plate geometry: cone angle, 4°; diameter, 2 cm; amplitude of strain at all frequencies 3%).

(A) Storage ($G'$) and loss ($G''$) moduli: closed symbols, $G'$; open symbols, $G''$; diamonds, F3; circles, F4; squares, F6; triangles, F8.

(B) Tangent of the loss angle. Symbols: diamonds, F3; circles, F4; squares, F6; triangles, F8. Taken from Cornec et al. (1994), with permission.
contained mostly gliadin monomers. With decreasing extractability, the fractions became enriched in glutenin polymers of medium and large size with the HMW subunits being concentrated in the largest, most insoluble polymers. The mechanical spectra of the fully hydrated fractions were also recorded in a dynamic assay in shear (Cornec et al., 1994). The viscoelastic properties of pure gliadin were too low to be measured with the Carrimed SL 100 stress rheometer but an increasing content of glutenin polymers was associated with increases in both the storage ($G'$) and loss ($G''$) moduli but with a decrease in the $G''/G'$ ratio (tangent $\delta$), indicating that the elasticity of the material increased (Figure 12). For all the fractions except those containing the lowest amount of glutenin, $G'$ was higher than $G''$ over the whole range of frequencies (with a cross-over of $G'$ and $G''$ being observed for gliadin-rich fractions, as shown for F3 in Figure 12). The frequency range corresponding to the elastic plateau was also shifted towards higher values when the content of glutenin polymers increased. This behaviour is typical of a transient network structure and the connectivity of the network, measured by the height of the elastic plateau ($G_N^0$), increased when the extractability of the fractions decreased. Thus, $G_N^0$ was between about 3000 and 30 000 N m$^{-2}$ for the glutenin-rich fractions and was strongly positively correlated with the content of large glutenin polymers (measured as the excluded peak in SE-HPLC), but not with that of medium-size glutenin polymers. Therefore, it was assumed that the density of transient cross-links in hydrated gluten was determined primarily by the proportion of the largest glutenin polymers. In this network, monomeric gliadins act as plasticizing elements (Cornec et al., 1994). Using a different fractionation procedure, Tsiami et al. (1997a, b) confirmed that the major variable affecting the rheological properties of gluten proteins, and particularly of glutenins, is the size of the concatenations.

B. INCORPORATION OF PROTEIN FRACTIONS INTO DOUGH

In other studies, different types of isolated gliadins or glutenin subunits have been incorporated into gluten and dough to discriminate between the effects of individual proteins and to relate functionality to particular structural features. The addition of isolated gliadins confirmed that monomeric prolamsins have a weakening effect on dough (Fido et al., 1997), by increasing the extensibility and decreasing resistance. However, the extent of the effect depended on the gliadin type, and conflicting results were reported by different authors (Fido et al., 1997; Uthayakamuran et al., 2001), with both molecular mass and hydrophobicity being reported to determine gliadin functionality. Such experiments are relatively easy to perform with gliadins because they are present in the flour as monomeric proteins. A
simple mixing after addition of the protein into a control flour is sufficient to observe the changes in dough properties induced by the added components. In the case of the glutenin subunits a more complicated procedure must be used, because the subunits must be incorporated by covalent disulphide bonds into glutenin polymers in order to express their functionality in the same manner as in the native gluten. Special procedures to reduce and reoxidize flour proteins were therefore developed and applied in solution (Schropp et al., 1995; Veraverbeke et al., 2000a, b) or in dough (Békés et al., 1994a; Uthayakumaran et al., 2000b). When reoxidation of HMW subunits was performed in solution, only partial polymerization was obtained, with the extent depending on protein concentration and type and on the concentration of oxidizing agent (Schropp et al., 1995; Veraverbeke et al., 2000a). About 20–40% of the initial subunits remained as monomers, with intramolecular instead of intermolecular bonds being formed. Some conflicting results were also reported concerning the respective oxidizing efficiencies of KBrO₃, KIO₃ and H₂O₂ (Schropp et al., 1995, Veraverbeke et al., 2000a, b). Although no differences in the degree or pattern of polymerization were observed when a mixture of subunits 1Bx5, 1Bx7, 1By9 and 1Dy10 was compared with a mixture of subunits 1Dx2, 1Bx6, 1By8 and 1Dy12 (Schropp et al., 1995), y-type subunits were found to be less highly polymerized than x-type subunits (Veraverbeke et al., 2000b) and subunit 1Dy10 was found to form the lowest proportion of polymers (Antes and Wieser, 2001b). Antes and Wieser (2001b) also noted that subunit 1Dx5, which contains an additional cysteine residue, yielded a higher proportion of polymers than subunit 1Bx7, whereas the opposite was noted by Veraverbeke et al. (2000a). It is also notable that LMW glutenin subunits showed a higher propensity to polymerize \textit{in vitro} than HMW subunits and that the polymers formed by HMW and LMW subunits did not differ in their size distribution (Veraverbeke et al., 2000b). These results are not consistent with those obtained with native gluten, where the largest glutenin polymers contain a higher proportion of HMW subunits (Payne and Corfield, 1979; MacRitchie, 1989; Lundh and MacRitchie, 1989), nor with results obtained with lines depleted in HMW subunits, where the absence of HMW subunits increased the extractability and decreased the average size of the glutenin polymers (Popineau et al., 1994; Gupta et al., 1995; Lefebvre et al., 2000). These conflicting results indicate that it is difficult to synthesize glutenin polymers \textit{in vitro} from isolated subunits and that it may be unwise to draw a conclusion about glutenin functionality in native gluten from such experiments.

Nevertheless, \textit{in vitro} polymerized glutenins have been incorporated into gluten and dough to test their effect on the rheological properties. The addition of glutenin polymers composed solely of HMW subunits clearly
increased the resistance of gluten and dough to extension, provided some free thiol groups were available to enable the added polymers to be linked to endogenous proteins (Schropp and Wieser, 1996; Antes and Wieser, 2001a). If no thiol groups were available, extensibility was decreased. Incorporation of polymers comprising LMW glutenin subunits also resulted in decreased extensibility, while the incorporation of mixed polymers decreased both the resistance and extensibility (Antes and Wieser, 2001a).

In other experiments, isolated glutenin subunits were directly incorporated into the dough using a partial reduction/reoxidation procedure, initially developed by Békés et al. (1994b). Provided the conditions were carefully controlled, reduction by dithiothreitol (DTT) could be completely reversed by addition of KIO$_3$, as judged by the mixing properties. However, the optimal conditions of reduction/reoxidation for mixing studies were recently shown to be unsuitable for extension and baking testing and specific procedures were therefore developed for each test (Uthayakumaran et al., 2000b). Extension tests were also shown to be more discriminating than mixing tests for determining the rheological properties of the treated doughs with the shape of the resistance vs. extensibility curve being especially sensitive to redox conditions. Even under the conditions determined as “optimal”, the overall shape of the curve after reduction and reoxidation was different from the original one, indicating that the structure of the protein network and the rheological behaviour of the dough were not restored to an identical condition, even if maximal resistance and extension were similar. Incorporation of HMW subunits always had a positive effect on strength, whereas the effects of incorporation of LMW subunits depended on the type of subunit added (Sissons et al., 1998). Increasing the HMW:LMW subunit ratio improved all parameters in the mixing test, with increased resistance and decreased extensibility of the dough. These changes were correlated with an increase in the amount of “unextractable polymeric proteins” (i.e. the largest glutenin polymers) (Uthayakumaran, 2000b).

C. INCORPORATION OF PURIFIED HMW SUBUNITS INTO DOUGH

Different HMW glutenin subunits have been incorporated into control doughs using reduction/reoxidation procedures. Incorporation of subunit 1Bx20 from the durum wheat cultivar Bidi 17 increased dough strength (i.e. increased mixing time and peak resistance and, decreased the breakdown in Mixograph tests). The amount of the largest glutenin polymers was also increased and the 1Bx20 subunit was recovered mainly from this polymer fraction (Békés et al., 1994b). On the other hand, the simple addition of the subunit (with no reduction and reoxidation to form inter-
chain disulphide bonds) resulted in a decrease in dough strength. This demonstrated that increasing the proportion of HMW glutenin subunit in glutenin polymers was associated with increases in their size and/or aggregation state, and significantly enhanced the technological properties of the flour. Using the same method, the effects of partially purified 1Dx and 1Dy subunits expressed in *E. coli* were investigated. Incorporation of the subunits increased dough strength, but the effect of 1Dx subunits was greater than that of 1Dy subunits. When incorporated in pairs, the most efficient associations were 1Dx + 1Dy, and subunit 1Dx5 combined with 1Dy10 resulted in a greater enhancement of strength than subunit 1Dx2 with 1Dy10 (Békés and Gras, 1994). The subunit pair 1Dx5 + 1Dy10 was also superior to the 1Dx2 + 1Dy12 pair. These results were confirmed by mixing, extension and baking tests on dough in which single subunits or

![Graph A](image1)

![Graph B](image2)

**FIG. 13.** Effect of incorporation of HMW glutenin subunits 1Bx7, 1By8, 1Dx5 and 1Dy10 on the mixing properties of two base flours, Banks and Hartog. The purified HMW glutenin subunits labeled on the abscissa were incorporated into the base flours using the reduction–oxidation procedure of Békés and Gras (1994). (A) Mixing time; (B) resistance of dough to breakdown. Taken from Uthayakumaran *et al.* (2000c), with permission.
pairs of subunits, extracted from flours or expressed in *E. coli*, were incorporated (Uthayakumaran *et al.*, 2000c) (Figure 13). These experiments showed that chromosome 1B-encoded subunits were less able to increase dough strength than chromosome 1D-encoded subunits. When single subunits were compared, subunit 1Dx5 had a greater effect than subunit 1Bx7 while subunit 1Dy10 had a greater effect than subunit 1By8. Synergy was also noted for the chromosome 1D-encoded subunits in that the subunit pairs 1Dx5 + 1Dy10 or 1Dx2 + 1Dy12 had greater positive effects than each single component incorporated at the same total concentration (Békés and Gras, 1994; Uthayakumaran *et al.*, 2000c). This was not the case for the chromosome 1B-encoded subunits 1Bx7 and 1By8. The greatest effect of subunits 1Dx5 + 1Dy10 was also demonstrated using baking tests (Uthayakumaran *et al.*, 2000c).

The results obtained with the subunit pairs are consistent with the presence in gluten of dimers composed of x- and y- subunits (see above) and also provide experimental support for the quality scores of glutenin subunits based on correlations between the HMW composition of genotypes and their technological properties (Payne *et al.*, 1987b; Branlard *et al.*, 1992).

**D. INCORPORATION OF HMW SUBUNIT PEPTIDES INTO DOUGH**

In order to determine whether the HMW subunit repetitive domain was alone able to affect the mixing properties of dough, Buonocore *et al.* (1998) expressed a series of repetitive peptides in *E. coli* and then incorporated them into dough using the 2 g Mixograph. The peptides had $M_r$ of about 58 000 and consisted of residues 103 to 643 of subunit 1Dx5 (comprising most of the repetitive domain), with the addition of short linking sequences at the $N$- and $C$-termini. In addition, a series of mutants were constructed with 0, 1 and 2 cysteine residues as substitutions close to the $N$- and/or $C$-termini.

When incorporated into dough, the peptide with two additional cysteine residues at the $N$- and $C$-termini (called 2 + 2) resulted in substantial increases in the mixing time and peak resistance (Figure 14), demonstrating that the nonrepetitive $N$- and $C$-terminal domains of the HMW subunits are not essential for the formation of the viscoelastic glutenin polymers.

**V. MANIPULATING HMW SUBUNIT COMPOSITION**

Although it is possible to obtain information on the functional properties of individual HMW subunits using *in vitro* approaches, it is important to
FIG. 14. The effect of an $M_r$ 58 000 repetitive peptide from subunit 1Dx5 on the mixing properties of dough.

A subclone encoding residues 103 to 643 from subunit 1Dx5 mutated to encode cysteine residues at positions 106, 112, 634 and 640 (A) was expressed in *E. coli*, purified (B) and incorporated into dough using the 2g Mixograph (C).

Tracks in part (B) are a, $M_r$ marker proteins; b, total proteins from uninduced *E. coli* cells; c, total proteins from *E. coli* cells after induction to express the $M_r$ 58 000 peptide (arrowed); d, the proteins extracted from induced cells of *E. coli* with 70% (v/v) ethanol and e, the purified $M_r$ 58 000 peptide. Taken from Buonocore *et al.* (1998) with permission.
also determine their properties when incorporated into glutenin polymers in vivo. In order to do this it is advantageous to compare the properties of individual subunits when expressed in the same genetic background. This can be achieved by classical crossing to produce near isogenic lines, or by transformation to insert additional genes. These approaches can be regarded as complementary, with the transgenic approach being used to generate variation beyond that which is available naturally, for example, to add additional copies of expressed genes or to express single genes or novel combinations of single genes which are usually inherited as tightly linked allelic pairs.

A. NEAR ISOGENIC LINES

The demonstration by Payne and coworkers (see review by Payne, 1987) that specific HMW subunit alleles are associated with good and poor breadmaking performance has led to their selection in plant breeding programmes. Although work was initially carried out on defined crosses (Payne et al., 1981a), the correlations demonstrated have since been confirmed and extended using collections of genotypes and different genetic stocks such as biotypes, null lines and near isogenic lines. Of these approaches, the development of near isogenic lines (NIL) is of greatest value for studies of structure–functionality relationships as they contain different combinations of HMW subunits transferred into a common genetic background by repeated back-crossing. A number of such series of lines are now available, in addition to near isogenic pairs such as the recently reported transfer of a novel 1Bx subunit into the breadwheat cultivar Fiorello (Margiotta et al., 2000).

1. The Sicco NIL

Payne et al. (1987a) have described the production of lines in the cultivar Sicco differing in their number (two to five) and composition of HMW subunits. These contained the following subunit combinations:

<table>
<thead>
<tr>
<th></th>
<th>Glu-A1</th>
<th>Glu-B1</th>
<th>Glu-D1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sicco</td>
<td>1</td>
<td>7 + 9</td>
<td>5 + 10</td>
</tr>
<tr>
<td>2 + 12</td>
<td>1</td>
<td>7 + 9</td>
<td>2 + 12</td>
</tr>
<tr>
<td>1Ax null</td>
<td>null</td>
<td>7 + 9</td>
<td>5 + 10</td>
</tr>
<tr>
<td>1D null</td>
<td>1</td>
<td>7 + 9</td>
<td>null</td>
</tr>
<tr>
<td>1Ax/1D null</td>
<td>null</td>
<td>7 + 9</td>
<td>null</td>
</tr>
</tbody>
</table>

Subsequently, Rogers et al. (1991) used the same Sicco background to
produce three additional NIL differing in the absence of x-type or y-type subunits. They had the following compositions:

<table>
<thead>
<tr>
<th></th>
<th>Glu-A1</th>
<th>Glu-B1</th>
<th>Glu-D1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sicco</td>
<td>1</td>
<td>7 + 9</td>
<td>5 + 10</td>
</tr>
<tr>
<td>1Dx null</td>
<td>1</td>
<td>7 + 9</td>
<td>null + 36</td>
</tr>
<tr>
<td>1Dy null</td>
<td>1</td>
<td>7 + 9</td>
<td>2 + null</td>
</tr>
<tr>
<td>1Bx null</td>
<td>1</td>
<td>null + 8</td>
<td>5 + 10</td>
</tr>
</tbody>
</table>

2. The Gabo NIL

Lawrence et al. (1988) combined null mutations at all three Glu-1 loci to develop NIL in the cultivar Gabo with subunit numbers ranging from zero to five, as listed below.

<table>
<thead>
<tr>
<th></th>
<th>Glu-A1</th>
<th>Glu-B1</th>
<th>Glu-D1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>17 + 18</td>
<td>5 + 10</td>
</tr>
<tr>
<td>null</td>
<td>1</td>
<td>17 + 18</td>
<td>5 + 10</td>
</tr>
<tr>
<td>1</td>
<td>null</td>
<td>17 + 18</td>
<td>5 + 10</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>null</td>
<td>null</td>
</tr>
<tr>
<td>null</td>
<td>1</td>
<td>17 + 18</td>
<td>null</td>
</tr>
<tr>
<td>null</td>
<td>null</td>
<td>null</td>
<td>5 + 10</td>
</tr>
<tr>
<td>1</td>
<td>null</td>
<td>null</td>
<td>null</td>
</tr>
<tr>
<td>null</td>
<td>null</td>
<td>null</td>
<td>null</td>
</tr>
</tbody>
</table>

3. The Galahad NIL

Payne and Seekings (1996) described a series of NIL containing only single 1B subunits (either 1Bx6, 1Bx7 or 1By8) in the cultivar Galahad. These four lines have been used for detailed studies of the functional properties of HMW subunits as discussed below. In addition, two more recently produced series of NIL will undoubtedly also prove to be valuable in this respect.

4. The Pegaso NIL

Margiotta et al. (2000) have reported the development of lines in the bread wheat cultivar Pegaso (Figure 15). They include lines expressing novel x-type and y-type subunits with higher and lower mobilities on SDS-PAGE than the more widely occurring alleles, which may reflect the presence of differences in the length of their repetitive domains. Furthermore, a Glu-A1 locus expressing genes for both x-type and y-type subunits was
introduced from the wild tetraploid wheat *T. dicoccoides*, allowing a NIL expressing six subunits to be produced.

5. Single subunit lines

Lafiandra *et al.* (2000) have reported the production of a series of lines expressing single x-type (1Ax1, 1Dx2, 1Bx7) or y-type (1By8, 1Dy10, 1Dy12) subunits in the background of Gabo, by combining spontaneous mutations identified in collections of old wheat varieties. Crossing of these lines will allow the properties of novel combinations of x-type and y-type subunits (e.g. 1Dx2 + 1Dy10) to be determined.

B. TRANSGENIC LINES

The development of methods for the transformation of wheat lagged behind those for most other major crops (including rice and maize), with the first fertile transformed plants being reported barely a decade ago (Vasil *et al.*, 1992). Nevertheless, wheat transformation has now been established in a number of laboratories world-wide, resulting in renewed interest in quality targets.

The most widely used wheat transformation system is based on direct gene transfer, the DNA being coated onto the surface of microscopic gold
particles and literally shot into the cells using high-pressure helium gas. It is necessary to use a recipient tissue that can be regenerated into a whole plant and it is usual to use immature embryos for bread wheat and either immature embryos or immature inflorescence explants for pasta wheat (see Barcelo et al., 2001). The exogenous DNA appears to integrate in a random fashion into the genome of cells of the recipient tissue, but only some cells are transformed. This could result after regeneration in a chimaeric plant containing a mixture of transformed and non-transformed cells. Consequently it is usual to also use a “selectible marker” gene which will usually co-integrate with the gene of interest and allow the selective regeneration of transformed cells. The most widely used selectable marker genes confer resistance to toxic herbicides or antibiotics, which can therefore be used to kill non-transformed cells. An alternative gene delivery system, based on using the bacterium *Agrobacterium tumefaciens* as a vector, has been applied to wheat (Cheng et al., 1997) but is not widely used.

The delayed development of wheat transformation systems meant that genes for HMW subunits and information on their relationship to grain processing quality were already available when reliable systems become available, with the result that the HMW subunits were the earliest target selected for the improvement of wheat by transformation.

The first success was reported by Blechl and Anderson (1996), who constructed a chimaeric gene encoding a hybrid subunit comprising residues 1 to 124 of the mature 1Dy10 subunit fused to residues 130 to 196.

### TABLE V

PUBLISHED REPORTS OF THE EXPRESSION OF HMW SUBUNIT GENES IN TRANSGENIC WHEAT

<table>
<thead>
<tr>
<th>Species</th>
<th>Line</th>
<th>Subunit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bread wheat</td>
<td>Bobwhite</td>
<td>1Dy10/1Dx5 hybrid</td>
<td>Blechl and Anderson (1996)</td>
</tr>
<tr>
<td>Bread wheat</td>
<td>Bobwhite</td>
<td>1Ax1</td>
<td>Altpeter et al. (1996)</td>
</tr>
<tr>
<td>Bread wheat</td>
<td>L88-6</td>
<td>1Ax1</td>
<td>Barro et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>L88-31</td>
<td>1Dx5</td>
<td></td>
</tr>
<tr>
<td>Bread wheat</td>
<td>Bobwhite</td>
<td>1Dx5+1Dy10</td>
<td>Anderson and Blechl (2000)</td>
</tr>
<tr>
<td>Bread wheat</td>
<td>Canon</td>
<td>1Ax1</td>
<td>Pastori et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>Cadenza</td>
<td>1Ax1</td>
<td></td>
</tr>
<tr>
<td>Bread wheat</td>
<td>Pro INTA</td>
<td>1Ax1</td>
<td>Alvarez et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>Federal</td>
<td>1Dx5</td>
<td></td>
</tr>
<tr>
<td>Bread wheat</td>
<td>L88-6</td>
<td>1Dx5 mutants</td>
<td>He et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>L88-31</td>
<td>1Dx5 mutants</td>
<td></td>
</tr>
<tr>
<td>Pasta wheat</td>
<td>L35</td>
<td>1Ax1</td>
<td>He et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>Ofanto</td>
<td>1Dx5</td>
<td></td>
</tr>
<tr>
<td>Tritordeum</td>
<td>Three lines</td>
<td>1Ax1</td>
<td>Rooke et al. (1999a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1Dx5</td>
<td></td>
</tr>
</tbody>
</table>
848 of subunit 1Dx5. This essentially combined the N-terminal domain of subunit 1Dy10 with the repetitive and C-terminal domains of 1Dx5. The novel subunit was readily resolved from the endogenous subunits present in the recipient cultivar (Bobwhite) by SDS-PAGE and analysis of seeds showed accumulation at levels comparable to those of the native proteins. However, subsequent studies (Shimoni et al., 1997) showed that the novel subunit formed circular monomeric structures stabilized by head-to-tail disulphide bonds rather than becoming incorporated into glutenin polymers.

Since then HMW subunit genes have been successfully expressed in a number of lines of bread and pasta wheat, including commercial cultivars as well as model lines (Table V). They include two mutant forms of subunit 1Dx5 in which the length of the repetitive domain has been increased or decreased to determine the effects on the gluten properties. Also, HMW subunit genes have been used to transform cultivars of durum wheat and lines of tritordeum, the latter being a novel cereal produced by...
combining the genomes of pasta wheat and the wild barley species *Hordeum chilense* (Martin *et al.*, 1999). In all cases expression levels up to or exceeding those of the endogenous genes have been reported, with expression being stable over a number of generations. However, both Blechl *et al.* (1998) and Alvarez *et al.* (2000) reported that some lines exhibited silencing or reduced expression of endogenous subunits, presumably via a co-suppression mechanism (see Barcelo *et al.*, 2001).

Of particular interest are some lines in which the levels of expression of the transgenes greatly exceed those of the endogenous genes. An example of this is shown in Figure 16 (Rooke *et al.*, 1999b). The line B73-6-1 was produced by transforming the line L88-6 (expressing subunits 1Ax1, 1Dx5, 1Bx17, 1By18, 1Dy10) with about 15 copies of the 1Dx5 transgene, resulting in a four-fold increase in the proportion of subunit 1Dx5 (from about 2.7 to 10.7% of the total seed proteins) and a 1.6-fold increase in total HMW subunits (from about 12.7 to 20.5% of the total). B73-6-1 and several other transgenic lines have now been grown in replicate field trials at two UK sites (Long Ashton Research Station near Bristol and Rothamsted near London) over four seasons (1998–2001) (Fido *et al.*, 2000; Popineau *et al.*, 2001). The effects of the transgenes on the functional properties are discussed in a later section.

VI. EXPERIMENTAL EVIDENCE FOR DIFFERENTIAL EFFECTS OF INDIVIDUAL HMW SUBUNITS ON MIXING AND RHEOLOGICAL PROPERTIES

*In vivo* evidence for differential effects of individual HMW subunits comes from comparative analyses of the functional properties of lines with different compositions of HMW subunits. These may be collections of cultivars, which led to the concept of “quality scores” discussed above, or more defined near isogenic and transgenic lines.

A. VARIATION BETWEEN CULTIVARS

The close association of subunits 1Dx5 + 1Dy10 with good breadmaking quality when compared with subunits 1Dx2 + 1Dy12 has been largely confirmed by analysis of a range of cultivars from around the world (Branlard and Dardevet, 1985; Cressey *et al.*, 1987; Campbell *et al.*, 1987; Payne *et al.*, 1988a; Ng and Bushuk, 1988; Lukow *et al.*, 1989; Mosleth and Uhlen, 1991; Dong *et al.*, 1991; Gupta *et al.*, 1991a; Dong *et al.*, 1992; Manley *et al.*, 1992). Kolster *et al.* (1991) have reported that the alleles present at the *Glu-D1* locus also modify the effects of alleles at the *Glu-
A1 and Glu-B1 loci on breadmaking quality. Thus, in the absence of subunits 1Dx5 + 1Dy10 correlations may be observed between Glu-A1 and Glu-B1 alleles and dough or gluten properties, as demonstrated by analysis of a collection of cultivars from southern Japan which contained subunits 1Dx2 + 1Dy12 or subunits 1Dx2.2 + 1Dy12 but not subunits 1Dx5 + 1Dy10 (Nagamine et al., 2000).

Furthermore, genetic variation in the LMW subunit composition must also be taken into account when predicting technological properties based on glutenin subunit composition. A study including 101 genotypes (48 from Australia and 53 from around the world) showed that allelic variation in both LMW and HMW glutenin subunit composition explained the rheological properties of dough as determined by extensimetry (Gupta et al., 1991a). In the world set of genotypes, most of the dough resistance was explained by the HMW subunits, with a marked difference between subunits 1Dx5 + 1Dy10 and 1Dx2 + 1Dy12. However, this was not observed in Australian lines, where the LMW subunit composition was more strongly correlated with resistance than the HMW subunit composition. This association was attributed to the presence of particular combinations of HMW and LMW subunit (Glu-1 and Glu-3) alleles in these genotypes, with high-quality Glu-1 alleles often being associated with low quality Glu-3 alleles.

Analysis of the dough properties of biotypes with identical gliadin compositions but different HMW subunit alleles indicated that those with the Glu-D1 subunits 1Dx5 + 1Dy10 had higher dough resistances than those with the subunits 1Dx3 + 1Dy12 and that these lines also contained a greater proportion of unextractable glutenin polymers (Gupta and MacRitchie, 1994). The effects of alleles at the Glu-A1 and Glu-B1 loci was much smaller (Lawrence et al., 1987). The dough properties and HMW glutenin subunit composition of the F3 population of the cross Nuri 70 (1Ax1, 1Bx17 + 1By18, 1Dx5 + 1Dy10) × UL 72 (1Ax null, 1Bx7 + 1By8, 1Dx2 + 1Dy12) were determined by Lagudah et al. (1988). No significant correlations were observed between dough properties and gliadin composition, but the resistance of the dough was strongly dependent on the HMW subunits alleles, especially those encoded at the Glu-D1 locus. In particular, the progeny containing subunits 1Dx5 + 1Dy10 showed higher dough resistance and lower resistance breakdown than those with subunits 1Dx2 + 1Dy12. On the other hand, no differences were observed between the Glu-B1-encoded subunit pairs 1Bx7 + 1By8 and 1Bx17 + 1By18. However, no relationship was found between HMW subunit composition and dough extensibility. Similar studies with other parental lines confirmed these results. Allelic variation at the Glu-D1 locus (i.e. subunits 1Dx5 + 1Dy10 vs 1Dx2 + 1Dy12)
explained most of the variation in the SDS sedimentation test values of random lines from the Cheyenne (1Ax2*, 1Bx7 + 1By9, 1Dx5 + 1Dy10) × MG 27116 (1Ax null, 1Bx7 + 1By8, 1Dx2 + 1Dy12) cross, whereas the Glu-B1 alleles had a much smaller effect (Benedetti et al., 1992). Analysis of recombinant inbred lines also showed that subunits 1Dx5 + 1Dy10 were associated with superior qualitative traits compared with subunits 1Dx2 + 1Dy12 (Rousset et al., 1992).

The loci encoding the HMW and LMW glutenin subunits have therefore been ranked as follows according to their contribution to the dough quality (essentially resistance) (Gupta et al., 1994): Glu-D1 > Glu-B1 > Glu-B3 > Glu-A3 > Glu-D3 = Glu-A1. However, additive and epistatic effects between the loci were also noted, and the differences in quality could only be assessed by taking into account the alleles present at the Glu-1 loci (encoding HMW subunits) and the Glu-3 loci (encoding LMW subunits) (Rousset et al., 1992; Gupta et al., 1994). It was also noted that factors other than the quantity of the HMW subunits were responsible for the effects of alleles at the Glu-D1 and Glu-B1 loci on the development time and the resistance of the doughs, in particular the size distribution of the polymeric proteins (Singh et al., 1990a, b; Gupta and MacRitchie, 1994), which was largely determined by allelic variation in the HMW (and LMW) glutenin subunits. Thus, lines with subunits 1Dx5 + 1Dy10 contained a higher proportion of unextractable polymers and showed a longer dough development time than other lines. Similarly, the presence of high molecular mass glutenin polymers and of unextractable polymeric glutenin proteins had previously been shown to be strongly correlated with dough strength (Dachkevitch and Autran, 1989; Singh et al., 1990b; Gupta et al., 1992, 1993). This indicates that the individual HMW subunits do not have the same propensity to polymerize, which could be the basis for the differences in dough properties associated with allelic variation in their composition. This is substantiated by preliminary studies of the in vitro polymerization of isolated subunits (Candler et al., 1996). Subunits encoded by the Glu-1 locus oxidized more slowly than subunits encoded by the Glu-D1 locus, with the x-type subunits oxidizing faster than the y-type for each locus. A synergistic effect was observed when x- and y-type subunits were mixed, accelerating the oxidation and leading to a higher proportion of large polymers.

Glutenin macropolymer (GMP) has been defined as the wheat protein fraction unextractable in 1.5% (w/v) SDS (Graveland et al., 1980). It contains principally glutenin polymers and its composition is strongly affected by dough mixing and resting (Hamer and Lichtendonk, 1987). The highest contents of GMP were found in flours, but mixing resulted in a significant decrease in the GMP content of dough and dough resting in
an increase. These changes in GMP content (i.e. in the extractability of glutenin polymers) were interpreted as arising from de-polymerization and re-polymerization of glutenin (Hamer and Lichtendonk, 1987). The contents of GMP in flours and doughs of 14 cultivars were also compared and related to the mechanical properties and breadmaking performance of doughs (Weegels et al., 1997a). The de-polymerization/re-polymerization during dough mixing and resting were observed in all cultivars. The GMP contents also influenced dough rheology, with the contents after a 45 min rest explaining about 90% of the dough resistance to extension. Globally, GMP contents were better related to quality parameters than the classical Osborne protein fractions (i.e. albumins, globulins, gliadins and glutenins).

GMP is composed of about 70% LMW and 30% HMW glutenin subunits (Weegels et al., 1995). However, each type of subunit was not affected to the same extent by de-polymerization and re-polymerization (Weegels et al., 1997b). After mixing, GMP contained a lower proportion of HMW subunits than flour GMP and a selective de-polymerization of x-type HMW subunits was also observed during mixing (Skerritt et al., 1999). However, subunit 1Dx5 was found to be particularly resistant to de-polymerization (Aussenac et al., 2001). HMW subunits were also more prone to become extractable through de-polymerization, but resting of the dough restored their initial concentrations in GMP. In this respect, the HMW subunits can be considered to be more reactive than the LMW glutenin subunits; this could be due either to their structures and conformations or to the conformations of the glutenin polymers. Furthermore, y-type subunits were re-incorporated in GMP more rapidly than x-type subunits during dough resting. As a consequence, GMP became more enriched in y-type subunits after glutenin re-polymerization (Weegels et al., 1997b). These data on the dynamics of glutenin polymers during dough processing showed that they undergo complex rearrangements in their composition and properties in dough.

B. NEAR ISOGENIC LINES

Direct experimental evidence for a functional relationship between dough or gluten properties and HMW subunits comes from analyses of the series of NIL.

The first important result from analysis of the Sicco isogenics was to show that the absolute amount of HMW subunit protein had a significant effect on the dough properties, with the absence of one or three subunits having significant effects on the SDS sedimentation values and breadmaking performance. The absence of subunit 1Ax had a small but significant negative effect on the quality parameters, whereas the absence
of subunits 1Dx5 + 1Dy10 had a major negative effect, as observed previously with two sister lines of wheat exhibiting contrasting bread-making properties (Payne et al., 1988b). The absence of the 1Ax and 1Dx + 1Dy subunits had an even more pronounced negative effect.

Furthermore, substitution of subunits 1Dx5 + 1Dy10 by subunits 1Dx2 + 1Dy12 gave less elastic dough (Payne et al., 1987a). This demonstrated that a qualitative factor, probably related to the specific structures of the individual subunits, was also involved in the determination of the dough elasticity. Further studies of gluten extracted from these Sicco NIL showed that absence of HMW subunits encoded by Glu-A1 and Glu-D1 decreased the total content of HMW subunits by about 50%. This increased the extractability of the glutenin, with the amounts of large glutenin polymers, as determined by sequential extraction and SE-HPLC, being reduced. The rheological properties of the hydrated gluten were also affected, with considerable change in both the storage and loss moduli (Popineau et al., 1994b) (Figure 17). The height of the elastic plateau dropped to less than 1/30 the value for the standard line and the $G''/G'$

![FIG. 17. The height of the elastic plateaux ($G_N^0$) of glutens extracted from near-isogenic lines of Sicco and Gabo wheats differing in their composition of HMW glutenin subunits encoded at the Glu-A1, Glu-B1 and Glu-D1 loci. Dynamic rheological assays in shear were performed at 20°C on glutens fully hydrated with water using a Carri-Med CSL 100 constant stress rheometer (cone-plate geometry: cone angle, 4°; diameter, 2 cm, amplitude of strain at all frequencies, 3%).

Sicco 5+10 (1Ax1, 1Bx7 + 1By9, 1Dx5 + 1Dy10); Sicco 2 + 12 (1Ax1, 1Bx7 + 1By9, 1Dx2 + 1Dy12); Sicco double null: (1A null, 1Bx7+1By9, null); Gabo 5+10 (1A null, 1B null, 1Dx5 + 1Dy10); Gabo 17 + 18 (1A null, 1Bx17 + 1By18, 1D null); Gabo triple null (1A null, 1B null, 1D null).]
increased four-fold, representing a dramatic collapse of gluten elasticity. The substitution of the subunit pair 1Dx5 + 1Dy10, normally present in the Sicco genotype, by the 1Dx2 + 1Dy12 pair did not modify the total amounts of HMW subunits or the concentrations of individual components. Nevertheless, the gluten in the 1Dx2 + 1Dy12 line contained a lower proportion of large glutenin polymers and the height of the elastic plateau was decreased five-fold. The relationship between the contents of the largest glutenin polymers in gluten fractions and the height of the viscoelastic plateau, previously shown in fractionation experiments (Cornec et al., 1994), was also valid with the Sicco NIL, showing that the HMW subunit composition determines gluten viscoelasticity by modifying the polymer size distribution and the aggregative properties of glutenin (Popineau et al., 1994b).

Analysis of the Gabo NIL (Lawrence et al., 1988) confirmed these data. The mixing time of the dough (determined by Mixograph analysis) was decreased when HMW subunits were absent, the shortest time being recorded for the triple null line lacking all of the HMW subunits. In this study, a quantitative effect of the HMW subunits was observed and no differences were found between the contributions of subunits 1Ax1, 1Dx5, 1Bx17, 1By18 and 1Dy10 to dough functionality. Complementary experiments on the same lines indicated, however, that the presence of subunits 1Dx5 + 1Dy10 resulted in a longer dough mixing time and higher maximum resistance than subunits 1Bx17 + 1By18 (Gupta et al., 1995). The viscoelastic properties of glutens extracted from four of these NIL (the control, 1A/1B null, 1A/1D null and triple null lines) were compared by dynamic assay in shear (Lefebvre et al., 2000). Similar behaviour to that of the Sicco NIL was observed (Figure 17). When HMW glutenin subunits were absent, the rheological behaviour of the gluten was drastically modified: the height of the viscoelastic plateau decreased to a value equal to only 1/250th of the plateau in the standard line, and the position of the plateau was shifted to lower frequencies. Glutenin polymers accounted for 35% of total proteins in this triple null line but they were composed only of LMW subunits and contained only 5% of unextractable glutenin. When only HMW subunits encoded by the Glu-B1 locus (1Bx17 + 1By18) were present, the height of the plateau was lowered to about one half the value in the standard line. Furthermore, gluten from the 1Dx5 + 1Dy10 NIL exhibited the same plateau value as the standard line (Lefebvre et al., 2000). The size distribution of the glutenin also depended on the number and the type of HMW subunits that were absent. Thus, the absence of all of the subunits resulted in a very large reduction in the proportion of unextractable glutenin polymers (i.e. those requiring sonication to be extracted) in the flour (Gupta et al., 1995),
and their quasi-absence from the gluten, whereas the presence of the subunits 1Dx5 + 1Dy10 resulted in a higher amount of unextractable polymers than in the line with subunits 1Bx17 + 1By18. This was in agreement with the rheological properties of the glutens (Hargreaves et al., 1996, Lefebvre et al., 2000). From these assays, it was concluded that HMW glutenin subunits are practically indispensable for the formation of large aggregative polymers and that these cannot be formed by LMW subunits alone. It can therefore be concluded that HMW subunits constitute the basis for gluten viscoelasticity, because polymers below a critical size limit cannot efficiently entangle and so cannot contribute to the gluten strength properties (Gupta and MacRitchie, 1994; Bangur et al., 1997). Furthermore, a minimum amount of large glutenin polymers is necessary in order to confer viscoelastic behaviour to gluten fractions (Cornec et al., 1994). In addition, the different alleles have different “viscoelastic potential”, which is higher for subunits 1Dx5 + 1Dy10 than for subunits 1Dx2 + 1Dx12 or 1Bx17 + 1By18 and is related to their ability to form aggregates or polymers. These differences must arise from structural features of the individual subunits such as the presence of an additional cysteine residue in the sequence of subunit 1Dx5 or to the regularity of the conformation of the repetitive domain (Anderson et al., 1989; Flavell et al., 1989; Goldsborough et al., 1989; Shewry et al., 1992). This is discussed in more detail in a later section.

In the studies reported above the effect of allelic variation was analysed for pairs of subunits encoded by the Glu-B1 and Glu-D1 loci. The contributions of individual x-type and y-type subunits was studied by Rogers et al. (1991) using the Sicco null lines. The two Glu-D1 subunits showed a much greater positive effect on gluten strength and dough quality than the y-type Glu-1B subunit, confirming the ranking of the loci reported previously (Gupta et al., 1994) (Table I). Although the 1Dx subunit appeared to be superior to the 1Dy subunit, this observation was not conclusive because allelic variation was superimposed on the deletion of subunits (Rogers et al., 1991). Further experiments on the Galahad NIL (Galahad 6, Galahad 7 and Galahad 8, containing only the 1B-encoded HMW subunits 6, 7 and 8, respectively) demonstrated that gluten containing only a single y-type subunit was less extensible and more sensitive to heat treatment (Payne and Seekings, 1996). This was attributed to the additional cysteine residues present in y-type subunits, which could allow more extensive cross-linking of glutenin polymers.

C. TRANSGENIC LINES

The creation of transgenic lines of wheat differing in their HMW subunit
composition makes it possible to determine the effects of individual subunits on the rheological and technological properties of wheat gluten and thus to interpret functionality in terms of protein structure. Transgenes encoding subunits 1Ax1 (containing two cysteines available for intermolecular disulphide bonds) and subunit 1Dx5 (with three cysteines available for intermolecular disulphide bonds) were therefore expressed in bread wheat (Barro et al., 1997, Rooke et al., 1999b), pasta (durum) wheat (He et al., 1999) and tritordeum (Rooke et al., 1999a).

The introduction of one (1Ax1) or two (1Ax1 + 1Dx5) transgenic HMW glutenin subunits in a Gabo NIL line (L88-31) containing only the Glu-B1-encoded subunits 17 and 18 resulted in progressive increases in the mixing time of dough (Barro et al., 1997), demonstrating that the subunits encoded by the transgenes were incorporated into the gluten structure in the same way as normal glutenin subunits and contributed to dough strength. In contrast, expression of subunit 1Ax1 in the cultivar Bobwhite showed only small effects on mixing time and loaf volume (Vasil et al., 2001), although this could be related to the fact that this cultivar contains the 1BL/1RS chromosome translocation, which results in “sticky dough” and poor mixing and baking performance. Similarly, although Anderson and Blechl (2000) reported that the expression of subunits 1Dx5 and 1Dy10 (presumably in the cultivar Bobwhite) resulted in greatly increased mixing time (from about 4 to 17 minutes), the peak resistance was greatly reduced.

The 1Ax1 and 1Dx5 transgenes were also expressed in durum wheat lines that lack the D genome associated with high gluten viscoelasticity and were also silent for the Glu-1A locus. The absence of subunits 1Ax1 and 1Dx5 from the donor durum wheat lines therefore made it easy to evaluate the technological effects of transgene expression. Both transgenic subunits increased the strength and stability of the dough, with the presence of subunit 1Dx5 resulting in an overstrong dough that could not be properly mixed under normal conditions. However, blending of the transgenic 1Dx5 flour with a weak flour resulted in fortification when added at medium doses (He et al., 1999). The same subunits were also expressed in tritordeum, a fertile amphiploid between wild barley and durum wheat, which is also generally unsuitable for breadmaking. These lines also lacked subunits 1Ax1 and 1Dx5. The expression of subunit 1Ax1 resulted only in a small increase in the dough strength, whereas the expression of the subunit 1Dx5 improved both the mixing time and the resistance breakdown (Rooke et al., 1999a), but this difference could have resulted partially from the different expression levels of the two subunits.

More detailed studies have been carried out on the effects of the 1Ax1 and 1Dx5 transgenes on Gabo NIL expressing HMW subunits 1Ax1,
1Bx17 + 1By18, 1 Dx5 + 1 Dy10 (the high-quality line, L88-6) and only subunits 1Bx17 + 1By18 (the low-quality line, L88-31). Over-expression of subunit 1Dx5 in the transgenic line B73-6-1 doubled the proportion of the HMW subunits in the total proteins, reaching 20%, and increased the concentration of subunit 1Dx5 by four-fold (Rooke et al., 1999b), but the gliadin/glutenin ratio was only slightly altered. Dough from this transgenic line did not develop properly under the condition of hydration and mixing speed (88 rpm) used in the 2g Mixograph test, whereas the control line L88-6 behaved normally. A high-speed mixing was necessary to form a continuous and cohesive dough. Blending experiments with a weak flour also indicated that the B73-6-1 line was overstrong (Rooke et al., 1999b).

Further experiments were carried out on the L88-6 and L88-31 lines and the transgenic lines B73-6-1 (1Dx5 in L88-6), B102-1-2 (1Ax1 in L88-31) and B72-8-11b (1Dx5 in L88-31) grown in the field in 1998, including protein fractionation and determination of the size distribution and rheological properties of extracted gluten (Popineau et al., 2001). Subunits 1Ax1 and 1Dx5 accounted for about 50 and 70% of the HMW subunits in the transformed lines, respectively, compared with 0% (1Ax1 in L88-31) and 26% (1Dx5 in L88-6) in the control lines. Overexpression of subunits

![Image](https://via.placeholder.com/150)

**FIG. 18.** The height of the viscoelastic plateau ($G_0^N$) of glutens extracted from control and transgenic lines of wheat. Dynamic rheological assays in shear were performed at 20°C on glutens fully hydrated with water using a Carri-Med CSL 100 constant stress rheometer (cone-plate geometry: cone angle, 4°; diameter, 2 cm; amplitude of strain at all frequencies, 3%).

L88-31: control line (1A null, 1Bx17 + 1By18, 1D null); B102-1-2: transformed line expressing subunit 1Ax1 transgene in the L88-31 background. B72-8-11b: transformed line expressing subunit 1Dx5 transgene in the L88-31 background. L88-6: control line (1Ax1, 1Bx17 + 1By18, 1Dx5 + 1Dy10); B73-6-1: transformed line expressing the subunit 1Dx5 transgene in the L88-6 background.
1Ax1 and 1Dx5 in the transgenic lines B102-1-2 and B72-8-11b doubled the proportion of the HMW subunits in the total proteins, when compared which their respective control lines. Clear differences were observed, however, between the effects of subunits 1Ax1 and 1Dx5 on gluten physicochemical properties, emphasizing that differences in subunit structure can influence their contribution to gluten structure and rheology.

Sequential extraction and SE-HPLC showed that the expression of the subunit 1Ax1 transgene increased glutenin aggregation, but did not appear to result in increased cross-linking by disulphide bonds. Thus, only the average size of glutenin polymers may have been increased. Gluten viscoelasticity was only moderately altered by the expression of the subunit 1Ax1 transgene (with slightly higher storage and loss moduli) (Figure 18), which mainly increased the dough resistance to elongation during mixing (Figure 19). In contrast, overexpression of subunit 1Dx5 generated very large and insoluble aggregates, probably through covalent cross-linking of polymers. As a result, the glutenin was only completely extracted after reduction of disulphide bonds. The connectivity and viscoelastic moduli of the gluten network were also greatly increased (Figure 18). This effect can be attributed primarily to the presence of an additional cysteine residue available for intermolecular cross-linking in subunit 1Dx5 as compared with subunit 1Ax1 (see Shewry et al., 1992). The very high gluten strength also resulted in abnormal dough mixing behaviour, irrespective of whether the genetic background was L88-6 or L88-31 (Figure 19). It can be postulated that an excess of subunit 1Dx5 modified the glutenin (gluten) structure and hindered the formation of a homogeneous protein network. Furthermore, subunit 1Dx5 is always expressed as a pair with subunit 1Dy10 and there is evidence that dimers between these two subunits, and between other x-type and y-type subunits, are present as “building blocks” in the glutenin polymers (Lawrence and Payne, 1983; Gao et al., 1992; Tao et al., 1992; Werner et al., 1992; Shani et al., 1994). Over-expression of subunit 1Dx5 in the absence of additional subunit 1Dy10 (or another y-type subunit) could therefore have resulted in extensive restructuring of the glutenin polymers, with important consequences for gluten strength and for the mixing and baking properties of dough.

VII. THE MOLECULAR BASIS FOR CORRELATIONS BETWEEN HMW SUBUNITS AND QUALITY

It is clear from the preceding sections that we now know a great deal about the amino acid sequences of individual HMW subunits, which has led to
FIG. 19. Analysis of the mixing properties of transgenic wheats expressing additional HMW subunits using the 2g Mixograph. (A) SDS-PAGE of the HMW subunits from a, L88-31: control line (1A null, 1Bx17 + 1By18, 1D null); b, B72-8-11b: transformed line expressing 1Dx5 subunit transgene in the L88-31 background; c, B102-1-2: transformed line expressing 1Ax1 subunit transgene in the L88-31 background; d, L88-6: control line (1Ax1, 1Bx17 + 1By18, 1Dx5 + 1Dy10); e, B73-6-1: transformed line expressing 1Dx5 subunit transgene in the L88-6 background. (B–F) are mixographs of (B) L88-31; (C) B102-1-2; (D) B72-8-11B; (E) L88-6; (F) B73-6-1. The resistance is given as torque % and the mixing time in seconds. Taken from Popineau et al. (2001), with permission.
the development of generalized models for their structures, interactions and role in gluten structure and properties. However, it has proved more difficult to identify the precise molecular basis for the differential effects of individual subunits on processing properties that have allowed “quality scores” to be assigned to them (Table I). We will now consider our current knowledge of the molecular basis for these effects.

A. PROTEIN AMOUNT

There is good evidence from several studies that the presence of a 1Ax subunit (1Ax1 or 1Ax2*) is associated with an increase in the total amount of HMW subunit protein present in the grain, by about 1.5 to 2.0% of the total grain proteins when compared with the null allele (Seilmieier et al., 1991; Halford et al., 1992). It is probable that this quantitative difference accounts for the quality score of 3 assigned to subunits 1Ax1 and 1Ax2*, as broad correlations between the total amount of HMW subunit protein and good processing properties have been reported by other workers (Payne et al., 1988a; Lawrence et al., 1988; Gupta et al., 1991b; Békés et al., 1994b; Popineau et al., 2001).

B. PROTEIN SEQUENCES

The availability of complete amino acid sequences for allelic pairs of 1Dx and 1Dy subunits associated with good (1Dx5 + 1Dy10) and poor (1Dx2 + 1Dy12) quality allows comparisons to be made to identify features which may relate to their different properties (Figure 20). Comparisons between the sequences of subunits 1Dy10 and 1Dy12 show that they have identical N- and C-terminal domains, but their repetitive domains differ by 12 single amino acid substitution and by the deletion of two hexapeptides and two adjacent residues in subunit 1Dy10 and of two adjacent residues in subunit 1Dy12. Although these differences are minor, they may nevertheless affect the structure and stability of the protein domain. Thus, Flavell et al. (1989) noted that the differences in sequence resulted in a higher proportion of consensus repeat motifs in subunit 1Dy10, which should result in a more regular pattern of β-turns, while Hickman (1995) predicted the presence of 130 β-turns present mainly in the repetitive domain of subunit 1Dy10 but only 125 β-turns in subunit 1Dy12 (Figure 20). This difference in regularity could affect the stability and intrinsic elasticity of the subunits and their ability to form “trains” in gluten.

Similar minor differences between the amino acid sequences of the repetitive domains of subunits 1Dx2 and 1Dx5 are also observed with 13 single amino acid substitutions and the deletion of two hexapeptides and
HMW SUBUNITS OF WHEAT GLUTENIN

Insertion of three nonapeptides and two hexapeptides in subunit 1Dx5. In addition, they differ in one single amino acid deletion in the N-terminal domain of subunit 1Dx2. These differences are predicted to have little effect on the $\beta$-turn structure, with 211 and 212 turns predicted to be present mainly in the repetitive domains of subunits 1Dx2 and 1Dx5, respectively (Figure 20).

**FIG. 20.** Alignment of the amino acid sequences of subunits 1Dx2, 1Dx5, 1Dy10 and 1Dy12. The lines indicate $\beta$-turns predicted by the method of Chou and Fasman (1978), using a significance level of $1 \times 10^{-4}$. 

insertion of three nonapeptides and two hexapeptides in subunit 1Dx5. In addition, they differ in one single amino acid deletion in the N-terminal domain of subunit 1Dx2. These differences are predicted to have little effect on the $\beta$-turn structure, with 211 and 212 turns predicted to be present mainly in the repetitive domains of subunits 1Dx2 and 1Dx5, respectively (Figure 20).
FIG. 21. Transverse urea gradient gel electrophoresis of a range of x-type (1Ax1, 1Ax2*, 1Dx2, 1Bx6, 1Bx14) and y-type (1By8, 1By15, 1Dy10, 1Dy12) HMW subunits. Taken from Lafiandra et al. (1999), with permission.
However, one of the single amino acid substitutions could have an impact on the interactions of the subunits in gluten. This is the substitution of a cysteine residue for serine at position 97 (at the N-terminal end of the repetitive domain) of subunit 1Dx5. This could result in the formation of more highly cross-linked, and hence more elastic, polymer. Evidence for this comes from the expression of the 1Dx5 transgene in developing grain, as discussed above.

C. STABILITY OF ALLELIC SUBUNITS

It is possible that the differences in the degree of conservation of the repeat motifs present in allelic subunits lead to differences in protein conformation and/or conformational stability that affect their functional properties. A simple way to determine conformational stability is by gel electrophoresis in a transverse gradient of 0–8 M urea, as described by Goldenberg and Creighton (1984). Data obtained with this technique can provide information on the thermodynamics and the kinetics of the unfolding process and demonstrate changes in stability following introduction of a given mutation in the protein (Goldenberg, 1992). In general, proteins that unfold in a single, cooperative, two-state transition exhibit an abrupt decrease in mobility, with a single inflection point at the midpoint of the transition. Studies on the unfolding behaviour of different allelic variants of HMW subunits have been carried out (Lafiandra et al., 1999), demonstrating different behaviour for x-type and y-type subunits (Figure 21). The x-type subunits generally show a broad unfolding pattern with no clear transition as the urea concentration increases, suggesting the existence of several conformational intermediates. In contrast, most y-type subunits show an abrupt decrease in mobility on unfolding, with a single inflection point at the midpoint of the transition indicative of a protein unfolding in a single, cooperative, two-state transition. The free energy values associated with the unfolding process were calculated for the y-type subunits, which showed a two-state transition. Different values were calculated for the allelic subunits 1Dy10 and 1Dy12, with the former showing greater stability. This is consistent with the differences in conservation of the repetitive domains of these two subunits, as discussed above, and may provide a partial explanation for association of the subunit pair 1Dx5 + 1Dy10 with good quality when compared with subunits 1Dx2 + 1Dy12.

D. SUBUNIT INTERACTIONS

These studies indicate, therefore, that both the structure and stability of subunit 1Dy10 and the cross-linking behaviour of subunit 1Dx5 may
contribute to the greater dough strength associated with subunits 1Dx5 + 1Dy10 compared with 1Dx2 + 1Dy12. However, it is also possible that specific interactions occur between the individual subunits of the allelic pairs (i.e. 1Dx5 with 1Dy10, 1Dx2 with 1Dy12), resulting in synergistic effects on quality.

One of the well-documented effects of adding water to high molecular weight subunits is the change in mobility observed by NMR. These observations have contributed to the development of the loop and train hypothesis discussed below (see p. 281). It seems logical, therefore, that any interactions between different subunits that affect the rheology should be reflected in changes in mobility. Gil et al. (2001) have compared the $^{13}$C CPMAS (cross-polarization magic angle spinning) spectra of subunits 1Dx5, 1Dx2, 1Dy10 and 1Dy12 separately and in combination. The CPMAS spectra are only sensitive to the immobile parts of the proteins. Therefore, the experiment directly measures only those parts of the protein that are not moving rapidly and therefore correspond to regions in which the hydrogen-bonded interprotein interactions are present (these are referred to as chains in the following section). Similar measurements of the amount of immobile material can also be made using proton NMR relaxation time measurements. (Belton et al., 1994). Figure 22 shows relaxation time data obtained for a mixture of high molecular weight subunits, together with average values for subunits 1Dx5 and 1Dy10 calculated using CPMAS data. While the scatter in both sets of data is

![Figure 22. Relationship between the proportion of mobile protein and the water content of HMW subunits. Triangles: data from Belton et al. (1994) calculated assuming that all populations of relaxation times less than or equal to 125 µs contributed to the immobile signal. Open squares: data from Gil et al. (2001) using the average figures for the subunits 1Dx5 + 1Dy10.](image-url)
high, the same trends are evident. The scatter arises, in part, because of the intrinsic difficulty of measurement, but also because the two methods of measurement are not exactly the same.

When different subunits are compared using the CPMAS method differences in behaviour are observed. Table VI compares the amounts of immobile protein measured for mixtures of subunits 1Dx5 + 1Dy10 and 1Dx2 + 1Dy12 with the predicted values arrived at by simply averaging the values of the two components in the mixture. It is difficult to estimate the errors in the data, but the variation between the predicted and measured values for subunits 1Dx5 + 1Dy10 is probably not significant. For the mixture of subunits 1Dx2 + 1Dy12 the differences are much larger and suggest that the sensitivity to water content has been greatly changed by interactions of the two subunits. In particular, at intermediate water contents the interactions between the two subunits appear to result in a higher population of trains (see p. 281). If this were the case it would seem to run counter to the observation that enhanced dough strength arises from interactions of the subunits 1Dx5 + 1Dy10 rather than subunits 1Dx2 + 1Dy12. This is an area where further work is needed to compare the molecular level information obtained from spectroscopy with the macroscopic rheological information.

VIII. THEORETICAL BASIS FOR PROPERTIES OF THE HMW SUBUNITS AND THEIR ROLE IN GLUTEN VISCOELASTICITY AND DOUGH MIXING

Much of the above discussion has focused on providing a biochemical and genetic explanation for the role of the HMW subunits in determining breadmaking quality. However, recent work allows us to develop more sophisticated biophysical models to explain the fundamental properties of

**TABLE VI**

<table>
<thead>
<tr>
<th>Water : protein ratio</th>
<th>1Dx5 + 1Dy10</th>
<th>1Dx5 + 1Dy10</th>
<th>1Dx2 + 1Dy12</th>
<th>1Dx2 + 1Dy12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Predicted value</td>
<td>measured value</td>
<td>predicted value</td>
<td>measured value</td>
</tr>
<tr>
<td>Dry</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.6</td>
<td>36</td>
<td>50</td>
<td>38</td>
<td>95</td>
</tr>
<tr>
<td>1.8</td>
<td>32</td>
<td>40</td>
<td>52</td>
<td>29</td>
</tr>
</tbody>
</table>

Data recalculated from Gil et al. (2001).
HMW subunits and the molecular basis for their role in viscoelasticity and mixing.

A. WHY ARE HMW SUBUNITS INSOLUBLE?

It is often stated that gluten proteins are insoluble in water or that particular prolamins are insoluble or soluble in specific solvents. Unfortunately, the terminology is often used with respect to some pragmatic measure such as the ability to dissolve the protein to a sufficient extent to carry out some biochemical analysis. However, in thermodynamic terms the solubility of a component in a liquid at equilibrium can be defined by the equilibrium condition:

$$\mu_S = \mu_{0L} + RT \ln a_L$$

(1)

where $\mu$ is the chemical potential of the component in the solid (S) or the liquid (L). The subscript 0 refers to the standard state of the component, while $a$ is the activity of the component. Thus, for a system at equilibrium with a pure solid $\mu_S = \mu_{0S}$, and

$$RT \ln a_L = \mu_{0S} - \mu_{0L}$$

(2)

with the term on the right-hand side being a constant. In practice it is very difficult to achieve this condition for proteins since the standard state is a crystalline solid. In general, therefore, the equation must be modified to take account of the fact that the solid is not in its standard state. Assigning an activity to the noncrystalline protein may achieve this, if pseudo-equilibrium between solid and liquid is assumed. Thus:

$$\mu_{0S} + RT \ln a_S = \mu_{0L} + RT \ln a_L$$

(3)

This equation reflects the importance of the activities in both the phases. The term $a_S$ is a measure of the deviation of the solid phase from its standard state, which for proteins will be a function of sample history. The solubility as measured, therefore, will depend on the state of the solid sample and should be measured in a steady state for defined and reproducible solids and solvents. Unfortunately, pragmatic biochemical observations are often treated as if they were thermodynamic measurements. Nevertheless, it is obvious that addition of excess water to gluten or native high molecular weight subunits does not result in the formation of a clear solution as is the case with soluble proteins such as lysozyme or bovine serum albumin, and it is therefore legitimate to infer that the
factors favouring the formation of a solution are insufficient in the case of gluten.

In order for a solution to form there must be net decrease in the free energy of the protein/solvent system on mixing. Conceptually one can imagine that two steps have to take place: the first is the formation of a liquid from the solid phase, and the second is the mixing of the liquid solute and the solvent. The total free energy change ($\Delta G_{\text{sol}}$) is the free energy of the formation of the liquid solute ($\Delta G_{\text{fus}}$) plus the free energy of formation of the mixture ($\Delta G_{\text{mix}}$)

$$\Delta G_{\text{sol}} = \Delta G_{\text{fus}} + \Delta G_{\text{mix}}$$

In order for solution to occur there must be net decrease of the free energy, i.e. $\Delta G_{\text{sol}}$ must be negative, $\Delta G_{\text{fus}}$ will be positive and thus for solution to occur $\Delta G_{\text{mix}}$ must be negative and greater in magnitude than $\Delta G_{\text{fus}}$. The magnitude of $\Delta G_{\text{fus}}$ will depend on the strength of the intermolecular interactions in the solid; if these are strong then solution will not be favoured. The importance of considering both terms seems to have been neglected by some authors. For example, Singh and MacRitchie (2001) only consider the Flory free energy of mixing a liquid polymer with a liquid solvent (Flory, 1953) and ignore the role of $\Delta G_{\text{fus}}$. If this logic were applied to the solubility of inorganic salts one would expect compounds such as barium sulphate or lithium fluoride to be readily soluble, as the free energies of hydration of the component ions are quite favourable. However, in both of these cases the very high energy of formation of the crystalline state results in lack of solubility.

Free energy contains both enthalpic and entropic contributions, and some authors have suggested that gluten proteins (including HMW subunits) are insoluble because the entropy of solution is unfavourable. Assumptions about the importance of entropic terms can arise because of the neglect of the $\Delta G_{\text{fus}}$ term (Singh and MacRitchie, 2001), or because of the assumption that hydrophobic interactions in gluten make it insoluble. If a substance is hydrophobic, its interactions with water cause an increase in the hydrogen bonding of the water. As this is a more ordered state, the entropy of the system decreases on solution. Because this is not compensated by any enthalpic interaction, solution is not favoured. This view of gluten protein solubility is also based on the assertion that gluten proteins are hydrophobic. However, consideration of the amino acid composition does not provide support for this. In fact, gluten proteins are very rich in hydrophilic amino acids. The hydrophilic/hydrophobic balance can also be calculated using published data for individual amino acids and typical amino acid composition of whole gluten and HMW subunits (see
### TABLE VII

**FREE ENERGIES OF HYDRATION FOR AMINO ACIDS IN GLIADINS, GLUTENINS, WHOLE GLUTEN AND AN AVERAGE OF THE AMINO ACID CONTENTS OF 314 PROTEINS**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Free energy of hydration (kcal/mol)</th>
<th>Mol% amino acid in gliadins</th>
<th>Mol% amino acid in glutenins</th>
<th>Mol% amino acids in 1Dx5</th>
<th>Mol% amino acids in 1Dy10</th>
<th>Average amino content of 314 proteins (mol%)</th>
<th>Free energy of hydration for an average protein (kcal/mol)</th>
<th>Free energy of hydration for gliadins (kcal/mol)</th>
<th>Free energy of hydration for glutenins (kcal/mol)</th>
<th>Free energy of hydration for 1Dx5 (kcal/mol)</th>
<th>Free energy of hydration for 1Dy10 (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>asp</td>
<td>-3.11</td>
<td>2.8</td>
<td>3.7</td>
<td>0.48</td>
<td>0.64</td>
<td>9.8</td>
<td>-30.478</td>
<td>-8.708</td>
<td>-11.507</td>
<td>-1.4928</td>
<td>-1.9904</td>
</tr>
<tr>
<td>thr</td>
<td>-1.69</td>
<td>2.4</td>
<td>3.5</td>
<td>2.9</td>
<td>3.83</td>
<td>6.1</td>
<td>-10.309</td>
<td>-4.056</td>
<td>-5.915</td>
<td>-4.901</td>
<td>-6.4727</td>
</tr>
<tr>
<td>ser</td>
<td>-2.36</td>
<td>6.1</td>
<td>7</td>
<td>5.68</td>
<td>6.7</td>
<td>7</td>
<td>-16.52</td>
<td>-14.396</td>
<td>-16.52</td>
<td>-13.4048</td>
<td>-15.812</td>
</tr>
<tr>
<td>gln</td>
<td>-3.15</td>
<td>34.5</td>
<td>28.9</td>
<td>37.97</td>
<td>35.57</td>
<td>9.9</td>
<td>-31.185</td>
<td>-108.675</td>
<td>-91.035</td>
<td>-119.606</td>
<td>-112.046</td>
</tr>
<tr>
<td>pro</td>
<td>0.23</td>
<td>16.2</td>
<td>11.9</td>
<td>13.8</td>
<td>11</td>
<td>5.2</td>
<td>1.196</td>
<td>3.726</td>
<td>2.737</td>
<td>3.174</td>
<td>2.53</td>
</tr>
<tr>
<td>gly</td>
<td>-0.23</td>
<td>3.1</td>
<td>7.5</td>
<td>20.07</td>
<td>18.02</td>
<td>8.4</td>
<td>-1.932</td>
<td>-0.713</td>
<td>-1.725</td>
<td>-4.6161</td>
<td>-4.1446</td>
</tr>
<tr>
<td>ala</td>
<td>-0.06</td>
<td>3.3</td>
<td>4.4</td>
<td>3.02</td>
<td>3.67</td>
<td>8.6</td>
<td>-0.516</td>
<td>-0.198</td>
<td>-0.264</td>
<td>-0.1812</td>
<td>-0.2202</td>
</tr>
<tr>
<td>cys</td>
<td>-0.27</td>
<td>3.3</td>
<td>2.6</td>
<td>0.61</td>
<td>1.12</td>
<td>2.9</td>
<td>-0.783</td>
<td>-0.891</td>
<td>-0.702</td>
<td>-0.1647</td>
<td>-0.3024</td>
</tr>
<tr>
<td>val</td>
<td>0.04</td>
<td>4.8</td>
<td>4.8</td>
<td>1.69</td>
<td>2.55</td>
<td>6.6</td>
<td>0.264</td>
<td>0.192</td>
<td>0.192</td>
<td>0.0676</td>
<td>0.102</td>
</tr>
<tr>
<td>met</td>
<td>-0.1</td>
<td>1.3</td>
<td>1.4</td>
<td>0.24</td>
<td>0.48</td>
<td>1.7</td>
<td>-0.17</td>
<td>-0.13</td>
<td>-0.14</td>
<td>-0.024</td>
<td>-0.048</td>
</tr>
<tr>
<td>ile</td>
<td>0.07</td>
<td>4.4</td>
<td>3.7</td>
<td>0.48</td>
<td>0.64</td>
<td>4.5</td>
<td>0.315</td>
<td>0.308</td>
<td>0.259</td>
<td>0.0336</td>
<td>0.0448</td>
</tr>
<tr>
<td>leu</td>
<td>0.07</td>
<td>6.9</td>
<td>6.5</td>
<td>4.36</td>
<td>3.83</td>
<td>7.4</td>
<td>0.518</td>
<td>0.483</td>
<td>0.455</td>
<td>0.3052</td>
<td>0.2681</td>
</tr>
<tr>
<td>tyr</td>
<td>-2.82</td>
<td>1.8</td>
<td>2.5</td>
<td>5.56</td>
<td>5.42</td>
<td>3.4</td>
<td>-9.588</td>
<td>-5.076</td>
<td>-7.05</td>
<td>-15.6792</td>
<td>-15.2844</td>
</tr>
<tr>
<td>phe</td>
<td>-0.28</td>
<td>4.3</td>
<td>3.6</td>
<td>0.29</td>
<td>0.32</td>
<td>3.6</td>
<td>-1.008</td>
<td>-1.204</td>
<td>-1.008</td>
<td>-0.0812</td>
<td>-0.0896</td>
</tr>
<tr>
<td>lys</td>
<td>-3.77</td>
<td>0.6</td>
<td>2</td>
<td>0.73</td>
<td>1.12</td>
<td>6.6</td>
<td>-24.882</td>
<td>-2.262</td>
<td>-7.54</td>
<td>-2.7521</td>
<td>-4.2224</td>
</tr>
<tr>
<td>his</td>
<td>-2.18</td>
<td>1.9</td>
<td>2</td>
<td>0.48</td>
<td>2.07</td>
<td>2</td>
<td>-4.36</td>
<td>-4.142</td>
<td>-4.36</td>
<td>-1.0464</td>
<td>-4.5126</td>
</tr>
<tr>
<td>trp</td>
<td>-0.88</td>
<td>0.4</td>
<td>1.3</td>
<td>1.09</td>
<td>0.96</td>
<td>1.3</td>
<td>-1.144</td>
<td>-0.352</td>
<td>-1.144</td>
<td>-0.9592</td>
<td>-0.8448</td>
</tr>
</tbody>
</table>

The data for this table are taken from Janssen (1992) (amino acid contents) and Oobtake and Tatsuo (1993) (hydration free energies).

1 cal = 4.18 J.
Table VII). The result is consistent with the calculations of Belton (1995) and shows that the free energies of hydration for HMW subunits are very close to the average value calculated from a sample of 314 proteins of $-164 \text{ kcal mol}^{-1}$ ($-686 \text{ kJ mol}^{-1}$). From this point of view, gluten and high molecular weight subunits are typical hydrophilic proteins. This is entirely consistent with the swelling behaviour of gluten in water (Grant et al., 1999). Furthermore, comparison of the behaviour of the truly hydrophobic protein elastin with HMW subunits (Belton et al., 1994) shows that the latter absorb water on heating whilst elastin ejects it, clearly demonstrating the hydrophilic nature of HMW subunits.

These results do not imply that hydrophobic interactions do not exist, but they do imply that the reason for lack of solubility cannot solely be hydrophobicity. Singh and MacRitchie (2001) have argued that the lack of charged groups may also contribute to the lack of solubility of gluten proteins. This is undoubtedly the case, but the authors fail in their analysis to take account of the importance of polar groups in contributing to polymer solubility. Polar groups such as OH groups can account for the solubility of many polysaccharides; gluten is rich in glutamine and thus polar NH$_2$ groups, which would be expected to interact favourably with water.

It is clear, therefore, that the insolubility of HMW subunit proteins needs some explanation. We consider that the answer lies mainly in the $\Delta G_{\text{fus}}$ term, not in the $\Delta G_{\text{mix}}$ term. The polypeptide polyglutamine is not soluble in water, but exists as an insoluble material with a $\beta$-sheet conformation. Similarly, Perutz (1996) has identified the formation of “polar zippers” formed between stretches of glutamine residues in pathological forms of human proteins. Recent modelling work (Belton et al., 2000) has suggested that the formation of intermolecular $\beta$-sheet involving glutamine residues present in HMW subunits may also occur. The hydrogen bonding pattern will also have the effect of placing the hydrophobic side chains within the interior of the protein. The role and importance of $\beta$-sheet formation will be discussed in more detail later, but for the present we note that the reason for the insolubility of the HMW subunits and other gluten proteins in water is probably the formation of strong inter- and intra-protein interactions rather than hydrophobic interactions with water.

The question still remains as to why it is possible to dissolve HMW subunits in alcohol–water mixtures, and in this case the answer may lie in the balance of hydrophobic and hydrophilic forces. The addition of alcohol will favour the interaction of the hydrophobic groups with the solvent; in addition alcohols are polar and so can interact with the amide groups of the glutamine residues. This interaction will clearly be less favourable than the interaction with water but, combined with favourable
interactions with hydrophobic side chains, may be enough to tip the balance in favour of solubility.

It is important to recognize that disulphide bonds interlink much of the protein present in doughs. This creates very large polymeric units (Lindsay and Skerrit, 1999) that appear to contain both HMW and LMW subunits, with the LMW subunits acting to both extend and terminate the chains of polymers. However, the extractability of glutenins from dough has been shown to be a strong function of mixing (Weegels et al., 1996) and it is assumed that extractability is related to polymer size. Clearly, if the polymer network is very extended, then the behaviour of the dough may be better understood as akin to a swollen polymer gel. However, it is important to note that the presence of the polymer does not alone account for the insolubility of the proteins in water since reduction of the disulphide bonds does not result in water solubility.

B. INTERACTIONS BETWEEN HMW SUBUNITS

As discussed above, consideration of the factors affecting subunit solubility suggest that hydrogen bonding may play a role. Early NMR relaxation time studies of C hordein, the barley homologue of the wheat \(\omega\)-gliadins, indicated that significant changes in the dynamics of the protein occurred as water was added, even though the protein did not dissolve (Belton and Gil, 1993; Belton et al., 1994). Similar results have since been observed with HMW subunits (Belton et al., 1995) and \(\omega\)-gliadins (Belton et al., 1998). The C hordeins and \(\omega\)-gliadins both resemble HMW subunits in containing repeat motifs that are rich in proline and glutamine residues. The similar behaviour of these proteins may therefore be attributed to competition with water for hydrogen bonding with glutamine. Increasing the water content results in a decrease in glutamine–glutamine hydrogen bonds and an increase in glutamine–water hydrogen bonds, leading to an increase in the mobility of the polymer chains.

A number of authors have used FT-IR spectroscopy to determine the structure of the proteins in gluten and related systems (Pezolet et al., 1992; Popineau et al., 1994b; Belton et al., 1995; Wellner et al., 1996; Grant et al., 1999; Gilbert et al., 2000). The broad conclusion from these studies is that gluten and the individual proteins contain \(\beta\)-sheet, \(\beta\)-turn and some \(\alpha\)-helix, the latter mainly being associated with nonrepetitive sequences in the C- and N-termini of the HMW subunits (see above). Detailed studies of the behaviour of HMW subunits and \(\omega\)-gliadins show that the proteins also undergo changes in conformation with water content. When dry, the proteins are largely disordered, but as water is added there are initially increases in the amounts of \(\beta\)-sheet and then increases in \(\beta\)-turns. More
recent work (Feeney et al., 2002) has shown that similar behaviour is observed with model peptides based on the repeat motifs of the HMW subunits.

The behaviour of the HMW subunits on hydration can be explained in terms of the formation and breaking of hydrogen bonds: at low water levels there are many intra- and inter-chain interactions. However, because there is very little molecular motion, the proteins remain in the disordered state that they adopt during sample preparation (typically freeze-drying). As more water is added, some molecular rearrangements to the lowest energy state will occur, which for glutamine-rich materials will be the $\beta$-sheet conformation. As yet more water is added, the water and the side chain amide residues will compete for hydrogen bond formation, decreasing the population of inter-chain $\beta$-sheet conformers and increasing the population of extended hydrated $\beta$-turn conformers. The transition from $\beta$-sheet to $\beta$-turn may be correlated with the changes in mobility observed by NMR; the $\beta$-sheet regions are of low mobility and the hydrated extended $\beta$-turns of high mobility.

C. HIGH MOLECULAR WEIGHT SUBUNITS AND DOUGH VISCOELASTICITY

This interpretation of the spectroscopic results has led to a model for the interactions of HMW subunits in doughs that offers an explanation for dough viscoelasticity. However, before considering this it is important to recognize that a theory of dough rheology is not a theory of breadmaking quality. Although appropriate dough mechanical properties are necessary for breadmaking, they are not sufficient. Bubble stability, oven spring, and the stabilization of structure by starch gelation depend on other factors. Theories of dough rheology can, therefore, only be expected to explain those observations that relate to the mechanical properties of dough.

Before considering theoretical models in more detail, it is important to clarify the thermodynamic basis of elasticity. It is often assumed that the elastic component of gluten viscoelasticity is purely entropic. This can arise because of a misunderstanding of the basic thermodynamics involved. For example, Ewart (1989) assumed that the second law of thermodynamics ensures that all spontaneous changes are entropy-driven. Although it is true that the entropy must increase in a spontaneous change for whole closed systems, this may occur because of enthalpic effects causing a rise in temperature. It is certainly not true for any individual component of that system, and if it were crystallization could not occur. The second law does not, therefore, imply that elasticity must be the result of entropic forces; the total free energy change is critical. In addition to these a priori reasons
for not assuming that elasticity is driven by entropy in gluten, recent studies by Tatham et al. (2001), in which the temperature coefficient of the elasticity of high molecular weight subunits was measured, have shown empirically that elasticity is not entropic.

Some of the key observations on the mechanical behaviour of dough are as follows:

1. Dough is viscoelastic.
2. The mechanical behaviour of dough is strongly dependent on water content.
3. The mechanical behaviour of dough is strongly dependent on the nature and concentration of HMW subunits present.
4. Mixing in D$_2$O rather than H$_2$O results in stronger dough.
5. Esterification of glutamine residues reduces the coherence and the resistance to extension of doughs.
6. The presence of oxidants and reducing agents affects dough rheology.
7. The resistance to extension of doughs reaches a maximum on mixing.

A reasonable starting assumption is that dough consists of a protein matrix in which are embedded starch granules. This matrix contains a cross-linked polymer network with some noncovalently bonded readily extractable proteins. The readily extractable proteins are unlikely to contribute to the elasticity of the dough since elasticity requires an interconnected network, albeit not necessarily a covalently bonded one. Elasticity therefore resides in the network. The network comprises both HMW and LMW subunits, which may interact with each other through covalent disulphide bonds and non-covalent interactions. Although the exact organization and role of the low molecular weight subunits in the polymer is not clear (Lindsay and Skerrit, 1999; Shewry et al., 2001), it is possible that they can act both as chain terminators and extenders. Our current knowledge of the disulphide bond structure of glutenin indicates that most disulphide bonds between subunits are end-to-end, although some branches also occur (see above). Located between these cross-links are the repetitive domains that interact with the repetitive domains of other proteins by hydrogen bonds. Interactions between HMW subunits will therefore tend to increase the cohesiveness of the dough, while interactions between HMW and LMW may increase or decrease cohesiveness, depending on the precise interactions. If the interaction results in the masking of the “interactive surface” of a HMW subunit, then the cohesiveness may be diminished. If, however, the external surface of the LMW subunit can hydrogen bond, then cohesiveness may be unaffected or increased. Interactions between HMW and LMW subunits could, therefore, affect dough rheology by both hydrogen bonding and disulphide interactions. This is illustrated in Figure 23.
Irrespective of the exact role of LMW subunits, the starting point for the theory of dough rheology is that elasticity originates mainly from interaction between the HMW subunits, although these may be mediated by the LMW subunits. The hydrogen bonded interactions between repeat regions (which we term chains) of these would be expected to be mediated by water. Thus, in the dry state strong interactions would be expected. Addition of water would result in competition between water and amino acid residues to form hydrogen bonds, breaking some of the inter-chain interactions. However, the density of glutamine residues and the proximity of the chains would result in many inter-chain interactions, and it is unlikely that all would be broken simultaneously. Two types of interaction will therefore be present in equilibrium in the hydrated system: hydrated regions, which, as above, are identified with mobile, extended $\beta$-turn structures, and regions of subunit interactions identified with rigid $\beta$-sheet structures. These two regions have been termed “loops and trains” (Belton, 1995, 1999a), in analogy with the interactions of polymers with surfaces (Dickinson, 1992). In the latter the polymer has many interactions with the surface, making it almost impossible to wash it from the surface once it has been absorbed. Even if the individual interactions
are quite weak, the statistical probability of all the interactions being broken simultaneously is very low. Polymers at interfaces thus exist in train regions where there are a number of interactions between the polymer and surface and solvated loop region. This is illustrated in Figure 24. The “loop and train” hypothesis has been supported by recent work of Alberti et al. (2001), who showed, using proton magic angle spinning NMR, that hydrated HMW subunits contained two types of glutamine residues. It is probable that one of these is associated with loops and the other with trains.

As mechanical extension is applied to the gluten network, the first effect will be to extend the loops, as this will require relatively little energy. Further extension will strain the loops and pull the β-sheet train regions apart, while even further extension may result in breakage of the inter-chain disulphide bonds. When the extending force is relaxed the tendency will be to restore the balance between loops and trains, since this is the equilibrium situation. If the time scale for extension is faster than the intrinsic relaxation rate of the polymers, then the application of mechanical force will result in the build up of stored elastic energy of the system. This is illustrated diagrammatically in Figure 25, in which the polymeric network is simplified and shown as a group of cross-linked linear polymers and the LMW subunits omitted.

The model leads to an explanation of some of the points made above. It explains why dough is viscoelastic (point 1) as it provides a mechanism for both extension and elastic recovery. The effects of water (point 2) may be seen in the changes that hydration causes in the ratio of loops to trains. Thus, increasing the water content will result in an increase in the loop to train ratio, which will make extension easier. However, the loop to train ratio cannot increase without limit as it is constrained by the disulphide cross-links that will keep the proteins in close proximity, by the solubility of the proteins which is in turn limited by the interchain interactions, and by breadmaking practice that typically maintains the water content of
dough at about 65%. Assuming that the water is evenly distributed between all components, this would result in about six water molecules per amino acid residue. A similar calculation has been reported by Ewart (1989). This is a very high protein concentration but it is still not high enough for glass formation at room temperature. The system is, therefore, in a rubbery state (Noel et al. 1995; Kalichevsky et al., 1992) and is liable to plasticization by water by the mechanism proposed. It should be noted that the term rubbery is used in this context to denote the opposite of glassy and does not imply that dough behaves according to rubber elasticity theory.

In the hypothesis outlined above the HMW subunits play a crucial role and are assumed to be responsible for most of the gluten viscoelasticity. This may seem surprising, as they only account for about 10% of the total grain protein. However, it is not unusual for the rheology of a food system to be governed by the behaviour of a minor component, and high

**FIG. 25.** Schematic depiction of changes in the polymer network in dough during extension. A two-dimensional network is shown for clarity; in reality the network is three-dimensional. (A) Network before extension; (B) the extended network; (C) the network after relaxation. Note that after relaxation some polymers have become detached from the network.
molecular mass polymers can radically change the rheology of systems even when concentrations are low. A good example of this phenomenon is agar, which at 1% (w/v) will transform liquid water to a solid self-supporting gel by formation of a continuous network. Thus, the crucial role of the HMW subunits, which account for only about 20% of the total glutenin subunits, is not surprising. Any change in the concentration of the HMW subunits or any changes that might make a difference to the ratio of loops to trains will therefore directly affect the mechanical properties of the system (point 3).

The role of the polymer interactions can also explain the effects of mixing in the presence of D$_2$O and of the esterification of glutamine residues. Deuterium would form stronger hydrogen bonds than hydrogen in the $\beta$-sheet regions, which would result in stronger interactions and stronger doughs. Conversely, the esterification of glutamine residues would reduce the likelihood of $\beta$-sheet formation and thus weaken doughs (points 4 and 5).

Oxidizing and reducing agents (point 6) would be expected to have effects on disulphide bonds, with oxidizing agents being assumed to increase the number of disulphide bonds and reducing agents to decrease them (Weegels $et$ $al.$, 1996). Thus, the effects of oxidizing and reducing agents may be through their effects on the connectivity of the network. However, both oxidizing and reducing agents also increase the extractability during mixing. It is probable that reducing agents increase the extractability by reducing the number of disulphide bonds. In contrast, increasing cross-links by oxidation would increase dough stiffness and work input. This could result in more bond breakage, resulting in a fragmented structure and thus increased protein extractability (Weegels $et$ $al.$, 1996). It should be noted that implicit in the interpretation of extractability measurements is the assumption that the controlling factor for extraction is the nature of the polymer network. However, as noted above, equilibrium considerations do not apply in such systems and the controlling factor may be the kinetics of the extraction process. This may depend on such factors as fissures and cracks in the dough improving solvent ingress and solute egress and the effective dough surface area. So far these factors do not appear to have been investigated.

**D. EXPLANATION FOR THE ROLE OF THE HMW SUBUNITS IN DOUGH MIXING**

In order to understand the maximum resistance observed in doughs during mixing (point 7), it is necessary to explore the process in a more quantitative manner using a simple kinetic model. Figure 25(A) shows a
diagrammatic representation of the network of polymers in dough. In this model the HMW subunit network is simplified to a network of polymers that are cross-linked at the ends and are elastic. The elasticity, as explained above, results from the tendency of the HMW subunit network to return to its equilibrium ratio of loops to trains after extension. After extension the rate of restoration of equilibrium is relatively slow compared with the extension rate in the mixer. Thus, if there are $N_0$ polymers in the network, on extension due to mixing, the network is stretched and there will be $N_S$ stretched polymers formed. The effect of mechanical extension of the stretched network is to break $N_B$ polymers away from the network with $N_U$ polymers remaining unstretched. If the system is left for a suitably long time after mixing it relaxes to its original unstretched state, but $N_B$ polymers remain broken from it.

Following the work of Gras et al. (2000), the best characterized mixing process is that of the Mixograph. They showed that the action of the Mixograph was purely extensional and that extension resulted in the storage of elastic energy in the dough. They reported the behaviour of three different wheat flours in the system. The flours showed the usual behaviour in reaching a maximum in resistance to extension, the height of which deceased with increased water content. Water content also affected the behaviour of the dough after resting, with the resistance to extension decreasing with water content and the degree of extension to break increasing. Longer mixing decreased the resistance to extension and decreased the degree of extension to break of doughs of constant water content, which were rested after different mixing times.

The effects of extension on the network shown in Figure 25 may for simplicity be modelled by first-order kinetics (these are, of course, not realistic for a mixing experiment, but the kinetics of the process are complex and currently not known). First-order models are, therefore, used to illustrate the principles involved and demonstrate that realistic models based on a network theory, combined with the loop and train model, can reproduce some of the features observed during mixing. In the following it is assumed that each revolution of the Mixograph results in the creation of stretched polymers and that the rate of relaxation of the polymers from their stretched state is slow compared with the rate of mixing. Thus, mixing stores mechanical energy in the dough. Further, it is assumed that the formation of the network and the uniform hydration of the dough are fast compared with the mixing process.

The latter assumption may not be true for all mixing processes and some of the observed increase in the resistance of the dough may be due to network build up. However, this would not explain the observation of maxima in dough resistance, merely a monotonic increase and levelling,
and for simplicity it is ignored here. Such an effect could be modelled, however, by simply adding a term for the rate of increase in the number of polymers in the network.

After $R$ revolutions the system can be described by the following equations:

\[
\frac{dN_S}{dR} = k_1 N_U - k_2 N_S \tag{5}
\]

\[
\frac{dN_U}{dR} = -k_1 N_U \tag{6}
\]

\[
\frac{dN_B}{dR} = k_2 N_S \tag{7}
\]

and

\[
N_U + N_S + N_B = N_0 \tag{8}
\]

where $k_1$ is the rate constant for the rate of creation of stretched polymers and $k_2$ is the rate constant for the rate of breakage of the polymers.

In order to relate these equations to resistance to extension, additional assumptions about the elasticity of the polymer networks need to be made. Resistance, $\rho$, is related to the number of polymers by the simple linear equation

\[
\rho = E_U N_U + E_S N_S + E_B N_B \tag{9}
\]

where $E$ is the resistance to extension per polymer unit.

The most important factor controlling the extensibility of the network will be the number of polymers it contains that are already extended, because as more polymers are extended the possibilities for further extension will decrease. Unextended polymers that are attached to the network will resist extension since they are coupled to it by trains and will have the natural resistance to extension of any polymer. Broken polymers will only contribute viscous drag to the system, thus $E_S > E_U > E_B$.

The variation of the numbers of the various types of polymers with the number of revolutions is shown in Figure 26. Clearly the number of stretched polymers and hence the resistance reaches a maximum. Figure 27 shows the effects of varying the rate constant for the formation of broken polymers. A large value of $k_2$ results in a sharp maximum whereas a low value results in a flattening of the curve. It may be expected that stiffer doughs will result in the input of more mechanical energy for dough extension and hence to more breakages in the polymer network.

The effects of water content may be understood in terms of the behaviour $E_U$ and $E_B$: as water content increases it would be expected that the loop to train ratio would increase. This would result in easier extension.
of the unstretched polymers and would therefore decrease the resistance to extension of the dough. This would in turn result in a lower maximum resistance, since the total resistance is that of the stretched polymers plus that of the other polymers. It would also result in a lower breakage rate of the polymers since less energy would be required to extend the dough. The
changes in resistance to extension after resting can also be understood. Energy input into the doughs will result in an increase in the number of broken polymers. After resting, the polymer network will relax to its equilibrium configuration but the number of polymers in the network will have decreased. This will result in decreased resistance to extension, as the number of polymers in the network available to be stretched will be lowered. In addition, there will be a reduced degree of extension to break since the network integrity will be reduced by the loss of polymers from it.

E. IS THE LOOP AND TRAIN HYPOTHESIS CONSISTENT WITH OTHER MODELS?

The combination of the loop and train model with a simple kinetic approach offers an explanation of the role of the HMW subunits in determining many of the phenomena observed in dough viscoelasticity and mixing. By recognizing the importance of network formation by covalent linkages and the role of hydrogen bonding in understanding of the elastic component of dough behaviour, a testable hypothesis is formulated that is consistent with both mechanical and spectroscopic behaviour. Some other theories of the mechanical behaviour of doughs have also been formulated. (For a thoughtful review of some earlier work see El-Dash, 1991.) Ewart (1989) has formulated a hypothesis that assumes that the “glutenin molecules” are arranged in chains of folded units; on stretching these units unfold and the elastic restoring force is said to be entropic. As pointed out above, this argument is based on a misunderstanding. The glutenin molecules are assumed to be oriented by the shearing process in dough and overlap between oriented molecules is suggested to augment noncovalent interactions between these molecules. This proposal is not dissimilar to the loop and train model in that it recognizes the role of noncovalent interactions and a role for the stretching of the molecules in elasticity. However, explicit mechanisms are lacking and, apart from the prediction of the effects of shear on orientation, little is offered in the way of testable predictions.

Singh and MacRitchie (2001) consider the applications of polymer science to the properties of gluten. Some of the problems of their approach have been discussed above. The core of their argument is that resistance to extension of the dough arises through chain entanglements. However, these are not the generalized “entanglements” of classical polymer theory in which interactions between polymers are represented by a constraining tube (Doi and Edwards, 1986; Belton, 1999b), but specific points at which it appears that the polymers form tangles analogous to those observed on the macroscopic scale in string. Slippage through these entanglements is
proposed to cause resistance to extension. The authors then consider the effects of molecular weight on slippage and the draw ratio of the material. The problem with this approach is that it is nonspecific; it does not explain why any polymer of suitable molecular weight does not behave like dough or why specific effects of D$_2$O and esterification are observed. The effects of water are discussed in terms of the glass transition temperature of polymer–water systems and it is suggested that zein proteins of maize do not show dough-like properties at room temperature because of glass transition effects. In fact, the glass transition temperatures of wheat gluten are below 20°C for gluten when it has greater than 15% water content (Kalichevsky et al., 1992; Noel et al., 1995) and similar figures are reported for zein (Madeka and Kokini, 1996). Arguments about a role for glass transitions in dough rheology seem therefore to be untenable.

El-Dash (1991) has proposed a mechanism of viscoelasticity that has some features in common with the loop and train model and with Ewart’s model. He emphasizes that the relative rarity of cysteine residues in glutenin ensures that disulphide interchange is unlikely to be a cause of network formation and assumes that the disulphide-bonded network pre-exists. Noncovalent interactions are considered to be responsible for holding coiled, but not necessarily ordered, molecules together in chains and sheets; these are interconnected by the relatively rare disulphide bridges. Mixing enhances noncovalent intermolecular interactions by bringing more molecules into contact with each other while continued mechanical stress causes unfolding of the coiled molecules. The recovery of the coiled state is the mechanism of elasticity. Interestingly, he points out that any polymeric system in which covalent cross-links exist together with noncovalent intermolecular interactions might be made to demonstrate similar viscoelasticity to gluten.

All of the other models proposed therefore offer some insights, but none of them have been sufficiently developed to make testable predictions about the nature of dough viscoelasticity or to generate testable hypotheses.

IX. CONCLUSIONS AND FUTURE PROSPECTS

The initial demonstration of correlations between HMW subunit composition and breadmaking quality of wheat stimulated a wide range of studies (biochemical, biophysical and genetic) that demonstrated that these proteins play a crucial role in determining the viscoelastic properties of hydrated gluten and the mixing properties of dough.

Furthermore, the relationship between the allelic composition of the HMW subunits and dough mechanical properties has been shown to result
from both quantitative (i.e. subunit amount) and qualitative effects, the
latter relating to differences in the structures and properties of individual
subunits. In this respect the glutamine-rich repeated sequence domain
may be particularly important in establishing inter-molecular interactions
that contribute to both cohesiveness and elasticity.

Although the primary and secondary structures of the HMW subunits
have been studied in some detail, we still know very little about their
higher-order structures. However, it is clear that the functional state of
gluten consists of proteins assembled into polymers or aggregates, stabilized
by both covalent disulphide bonds and noncovalent forces (principally
hydrogen bonds). These protein assemblies are essential for the
expression of the mechanical properties; isolated HMW subunits are not
viscoelastic. Despite the paucity of our knowledge of the structures of
these assemblies, it is possible to propose a plausible physicochemical
mechanism for the role of the HMW subunits in gluten viscoelasticity,
based on studies of the structures and interactions of isolated subunits.

In addition to the genetic effects discussed above, it is clear that
environmental factors also affect the structure and properties of the gluten
polymers and hence grain processing quality. This results in extensive
year-to-year and site-to-site variation, posing problems for the grain
utilizing industries. Furthermore, there is also evidence for the existence of
genotype × environment (G × E) interactions, meaning that cultivars may
be differentially affected by environmental factors.

The elucidation of the detailed structures and properties of the glutenin
polymers is clearly an important goal for wheat scientists. This will not
only require the analysis of glutenin polymers isolated from dough, but
also the determination of their mechanisms of synthesis and its regulation,
their interactions with other dough components (notably lipids, starch and
arabinoxylans) and dynamic aspects of their changes in structure during
grain maturation and dough mixing. This is clearly a formidable challenge,
but one that can be faced using the combination of modern genetic,
molecular, biochemical and biophysical approaches discussed above.

ACKNOWLEDGEMENTS

IACR receives grant-aided support from the Biotechnology and
Biological Sciences Research Council of the United Kingdom.

Much of the work carried out in the authors’ laboratories was supported
by the European Union FAIR program CT96-1176 “Improving the
Quality of EU Wheats for the Food Industry – EUROWHEAT”.

REFERENCES


Rooke, L., Békés, F., Fido, R., Barro, F., Gras, P., Tatham, A. S., Barcelo, P., Lazzeri, P. and


INDEX

A
Acetylintermedine, 72, 75
Acetyllycosamine, 72, 75
Acoustic impedance, 117–118
Acoustic signal generator, 122
Actin–DNA cross-links, pyrrolizidine alkaloid antimitotic activity, 90–91, 92
Adenostyles alliariae (Alpendost), 76
Aegilops cylindrica, 228
Aflatoxins, 198
Agrobacterium tumefaciens transformation vector, 254
Alfalfa sprouts, ozone treatment, 196
Alginate gels, ultrasonic analysis, 152
Allicylobacillus acidocaldarius, 197
Alkanes, ultrasonic phase transition monitoring, 141
Alpendost (Adenostyles alliariae), 76
\(\alpha\)-gliadins, 222
\(\alpha\)-trinositol
\((\text{inositol}(1,2,6)\text{trisphosphate}), 28, 40\)
Aluminium–inositol phosphate interactions, 32, 36
Animal products, inositol phosphate content, 26–27
Anticaking agent, gaseous ozone treatment, 179–180, 198
Anticancer activity, inositol phosphates, 37, 38–39
Antioxidants
inositol phosphates, 28, 29
anticancer activity, 39
ozone interactions, 177
AP-2 (adapter protein 2), 25, 30, 38
AP-3 (adapter protein 3), 30
Apple juice, fruit washing water-related disease outbreaks, 169, 195
Apples, ozone treatment, 192, 195
of washing water, 190
Arabidopsis
Escherichia coli phytase transgenic expression, 34
phytate (inositol hexakisphosphate) synthetic pathway, 8
Argon, ozone combined sanitization procedures, 201
Aspergillus fumigatus, phytase transgenic expression, 34
Aspergillus niger, 11, 12, 36
phytase, 20
Asteraceae (Compositae), pyrrolizidine alkaloids, 65
Avocado, inositol phosphate content, 23

B
Bacillus cereus, 186
Bacillus spp., 186, 198
Bacillus stearothermophilus, 186, 187
Bacillus subtilis, 187, 199, 202
phytase, 12
Barley
inositol phosphate degradation during processing, 21
malting, 22
phytases, 9
reduced phytate gene mutations, 34
Beet root, inositol phosphate content, 23
β-gliadins, 222
Betony (Pedicularis), 77
BIOTEST, 138
Black beans, inositol phosphate degradation during processing, 21, 22
Blazing star (Liatris punctata), 77
Boraginaceae, pyrrolizidine alkaloids, 65
Botrytis cinerea, 181, 191
Bread, ultrasonic texture sensing, 146, 147
Breadmaking
inositol phosphates degradation, 16, 20
see also Glutenin high molecular weight subunits
Broccoli, ozone treatment, 192
Brussel sprouts, chlorination, 188

C
Cabbage
chlorination, 188
inositol phosphate content, 23
Cachana (Liatris punctata), 77
Cadmium–inositol phosphate interactions, 37
Calcium, inositol phosphate effects, 27, 29, 31
absorption impairment, 32, 35
inositol(1,4,5)P3 signaling, 27–28
Campylobacter spp., 193
Canning
filling operations, 130
phytate degradation, 16
Capillary chromatography, inositol phosphates, 6
Carbon dioxide, ozone combined sanitation procedures, 201–202
Cardiopulmonary toxicity, pyrrolizidine alkaloids, 80–81
Carrots, inositol phosphate content, 23
Casein, ultrasonic micelle particle sizing, 150
Castellija rhexifolia, 77
Castellija sp. (Indian paint brush), 77
Cauliflower, ozone treatment, 192
Celery, inositol phosphate content, 23
Cereal grains
inositol phosphates, 5–6
degradation during processing, 16, 21–22
ozone treatment, 198
pyrrolizidine alkaloids contamination, 68, 69–70
Cheese
ultrasonic texture sensing, 146, 147
ultrasonic velocity changes during melting, 142
Chickpeas, inositol phosphate degradation during malting, 22
Chinese millet, phytate content, 13
Chlorine dioxide sanitization, 188, 189
Chlorine sanitization, 169–170, 188–189
ozone combined procedures, 200–201
Chromium–inositol phosphate interactions, 36
Cider
fruit washing water-related disease outbreaks, 169, 195
pasteurization recommendations, 195
Coatomer, 30
Cobalt–inositol phosphate interactions, 36
Coltsfoot (Tussilago farfara), 76, 91
Comfrey (Symphytum), 74–76, 91
Commelina communis, phytate (inositol hexakisphosphate) synthetic pathway, 8
Compositae (Asteraceae), pyrrolizidine alkaloids, 65
Compressibility measurements, polymer hydration analysis, 151–152
Cookies, ultrasonic texture sensing, 146
Copper
hepatotoxic synergism with pyrrolizidine alkaloids, 79
phytate (inositol hexakisphosphate) interactions, 32, 35–36, 40
Corn
bran/flour phytate content, 16
inositol phosphate degradation during processing, 21
Coughwort (Tussilago farfara), 76
Crotalaria, 80
contamination of staple foods, 69, 70
prevention, 91
Crotalaria fulva, 74, 91
Cryptosporidium parvum, 169, 189
Cucumbers, ozone/carbon dioxide mixed gas treatment, 202
Cyclospora cayetanensis, 169
CYP 2C11, 82
CYP 3A, 82
CYP 3A4, 82
Cytochromes P450, pyrrolizidine alkaloids metabolism, 81, 82

D
Dairy products, ultrasonic composition sensors, 138
*Dictyostelium*
phytase, 12
phytate (inositol hexakisphosphate) synthetic pathway, 7
Dietary Supplement Health and Education Act (1994), 74, 91
Digital storage oscilloscope, 122–123
DNA cross-links
formation during pyrrolizidine alkaloid metabolism, 83, 88–90
DNA–protein cross-links, 90, 93
DNA repair, inositol phosphate requirement, 31
Doppler ultrasound flowmeters, 131–132
viscosity determination, 148, 149

E
Echimidine, 71, 72, 75
*Echium plantagineum* (Patternson’s Curse; Salvation Jane), 72
Echiumine, 72
Eggs
ozone inactivation of microbial contaminants, 186, 193–194
pyrrolizidine alkaloid concentrations, 73, 91
Elastography, carcass grading, 154
Elephant Head (*Pedicularis*), 77
Emulsions, ultrasonic characterization, 112, 114–116, 158
floculated emulsions, 116, 150
imaging, 154
particle sizing, 115–116, 150
practical applications, 150
Epi-inositol, 37
*Escherichia coli*, 178, 180, 183, 193
phytase, 9, 10, 11, 21
transgenic expression in *Arabidopsis*, 34
*Escherichia coli* O157:H7, 169, 178, 181, 188, 190, 195, 203
Europine, 73

F
Fabaceae (Leguminosae), pyrrolizidine alkaloids, 62
Fermentation, phytate degradation, 9, 16, 20, 22
Fish
ozone treatment, 197–198
ultrasonic composition sensors, 138
ultrasonic microscopy for parasite detection, 156
Flavin-containing monooxygenases, 82
*Flavobacterium aquatile*, 180
*Flavobacterium branchiophilum*, 181, 190
Fluid flow, ultrasonic sensing, 130–132
Doppler flowmeters, 131–132
transit-time mode, 130–131
vortex shedding, 132
Foams, ultrasonic analysis, 150–151
Foil pouches, ultrasonic leak detection, 154
Food processing equipment, ozone treatment, 192
Foreign bodies, ultrasonic detection, 151
Fourier transform infrared (FT-IR) spectroscopy, gluten proteins, 239, 278
Fruit
chlorine treatment, 188
inositol phosphates, 22–23
ozone treatment, 190, 191, 192, 194–195
pesticide residues reduction, 199
texture (rheological properties), ultrasonic sensing, 142
washing water-related disease outbreaks, 169, 194
Fruit juices
Love wave-based component analysis, 153
ozone treatment of ingredients, 196–197
Fulvine, 71, 74

G
Gabo near isogenic line, 252
dough properties, 260, 261
transgenic HMW glutenin subunit studies, 263–264
Galahad near isogenic line, 252
γ-gliadins, 222
Garlic, ozone treatment, 198
Gelation, ultrasonic analysis, 152
Gliadins, 221
dough viscoelasticity
gluten fractionation/reconstitution
experiments, 242, 243, 245
protein fraction incorporation studies,
245–246
electrophoretic separation, 222, 223
structural studies, 278
Glu-A1, 224, 226, 256–257, 258, 260, 263
near isogenic lines, 251, 252
Glu-A3, 258
Glu-B1, 224, 226, 257, 258, 262, 263
near isogenic lines, 251, 252
Glu-B3, 258
Glu-D1, 224, 226, 256, 257, 258, 260, 262
near isogenic lines, 251, 252
Glu-D3, 258
Glutathione metabolism, 79
Glutathione S-transferase, 83
Gluten
insolubility, thermodynamic aspects,
274–275, 276
macropolymer, 258–259
dough performance relationship, 259
viscoelasticity, 279–280
Glutenin high molecular weight subunits,
222, 223
amino acid sequences, 228-233
atomic force microscopy, 233, 235
composition manipulation, 249, 251–256
cross-link formation
disulphide bonds, 240-242, 278, 284
low molecular weight subunit
branches, 240, 242, 278
mechanical behaviour of dough, 280
transgenic lines, 265
tyrosine bonds, 242
dough viscoelasticity influence, 242–249
dough mixing process, 284–288
gluten fractionation/reconstitution
experiments, 242–245
in vitro polymerization, 246
‘loop and train’ hypothesis, 281–283,
284, 285, 286, 288–289
macropolymer interactions, 280–281
protein fraction incorporation studies,
245–247
purified subunits incorporation into
dough, 247–249
subunit peptides incorporation into
dough, 249, 250
theoretical models, 279–284
genetics, 224–226
grain breadmaking quality relationship,
226–228, 259–265, 266
biophysical models, 273–289
gluten macropolymer composition, 259
molecular basis, 265, 267–273
quality scoring system, 227
ranking of subunit loci, 258
relation to dough rheology, 279
variation between cultivars, 256–259
hydration-related molecular
rearrangements, 278–279, 284
insolubility, thermodynamic aspects,
274–278
mixing/rheological properties influence,
256-265
molecular basis of processing properties
amino acid sequences, 267–269, 271
subunit interactions, 271–273
subunit stability, 270, 271
total amount of protein, 267
molecular dimensions, 233, 234
near isogenic lines, 251–253
dough quality differences, 259–262
Gabo, 252, 260, 261
Galahad, 252
Pegaso, 252–253
Sicco, 251–252, 259–261
single subunit, 253
polymorphisms, 224–226
SDS-polyacrylamide gel
electrophoresis, 224, 225
structure
repetitive domains, 237–239
terminal domains, 234, 236–237
transgenic lines, 253–256
dough quality differences, 262–265,
266
Glutenin low molecular weight subunits,
222, 223
B-type, 222
C-type, 222
cross-links, 240, 242, 278
mechanical behaviour of dough, 280,
281
D-type, 222
dough viscoelasticity, incorporation
studies, 247
genetic variation, influence on
technological properties, 257, 258
nomenclature, 2–3
nutritional importance, 32–41, 42
health disorders prevention, 36–41
mineral bioavailability influence, 32–36, 42
receptors, 27, 28
seed contents, 12–23
processed foods, 16–22
whole raw seeds, 12–15
signaling activities, 7, 27, 28, 29
synthesis, 7–8
animal cells, 7–8
microorganisms, 7
plants, 8
Inositol pyrophosphates, 31
Inositol(1,2,6)trisphosphate (alpha-trinositol), 28, 40
Inositol(1,4,5)trisphosphate (Ins(1,4,5)PU3u), 5, 7
animal cells, 26
receptors (calcium channels), 27–28
signaling function, 27–28
plant cells, 23
Intermedine, 71, 72, 75, 77
Iron, inositol phosphates binding, 28–29
absorption impairment, 32, 33–34, 40–41
low-phytic acid grains, 34–35
anticancer activity, 38–39
overall dietary absorption impact, 40–41, 42

J
Jack mackerel, ozone treatment, 197
Jacobine, 72
Jacoline, 70, 72
Jaconine, 72
Jacozone, 72
Japanese flounder, ozone treatment, 197

K
Kimchi preparation, 192

L
*Lactobacillus* *leichmannii*, 203
*Lactobacillus* *plantarum*, 22
Lasiocarpine, 71, 73
Lead–phytate (inositol hexakisphosphate) interactions, 32
Legumes, phytate degradation during processing, 16
Leguminosae (Fabaceae), pyrrolizidine alkaloids, 62
Lentils, inositol phosphates degradation, 20, 22
Lettuce
chlorination, 188
ozone treatment, 192, 195–196, 206
*Leuconostoc mesenteroides*, 188
Level sensors, ultrasonic, 129–130
*Liatris punctata* (cachana; grey feather; blazing star), 77
Lily pollen phytase, 11
*Listeria monocytogenes*, 169, 188, 193, 203
Lousewort (*Pedicularis*), 77
Love wave sensors, 128–129, 147
arrays, 129
smart tongue and nose, 153
Love waves, 104
Low-phytic acid grains, 34–35
Lupin, inositol phosphates degradation, 22
Lycopsamine, 71, 72, 75, 77, 78

M
Magnesium absorption, phytate (inositol hexakisphosphate) impairment, 32, 35
Maize
phytases, 9
phytate (inositol hexakisphosphate), 9
degradation during processing, 22
reduced content gene mutations, 34
Malting, inositol phosphates degradation, 22
Manganese absorption, inositol phosphate effects, 36
Meat
elastographic carcass grading, 154
foodborne disease outbreaks, 169
ozone treatment, 186, 190, 191, 192, 193–194
protein ultrasonic analysis, 152–153
pyrrolizidine alkaloid residues, 73
sanitizing agents, 189
ultrasonic composition sensors, 138
ultrasonic imaging of carcasses, 154
Megalocytosis, pyrrolizidine alkaloid-induced, 88, 89
Mercury–inositol phosphate interactions, 32, 36
INDEX

Micrococcus spp., 198
Milk
contamination with pyrrolizidine alkaloids, 70, 72, 91
ultrasonic analysis
aggregates, 152
fat globule particle sizing, 150
imaging for spoilage measurement, 154
Mineral bioavailability, inositol phosphate influences, 32-36
Mixograph, 285
Molybdenum–inositol phosphate interactions, 36
Monocrotaline, 66, 67, 78, 79, 80, 88
Mucor piriformis, 181
Multiple inositol polyphosphate phosphatase (MIPP), 11, 12
Multiple scattering theory, 113–116
Mung bean, phytate (inositol hexakisphosphate) synthetic pathway, 8
Mussels, ozone treatment, 197
Mycobacterium fortuitum, 173
Myelin proteolipid protein, 30
Myo-inositol, 3, 36
anticancer activity, 37, 38
food sources, 36–37
plasma lipid effects, 40

N
Necic acids, 66
Neosartorya fischeri, 197
Neper (Np), 106
Nickel–phytate (inositol hexakisphosphate) interactions, 32, 36
Nuclear magnetic resonance (NMR) spectroscopy
high molecular weight glutenin subunits, 272–273, 279, 282
domain structures, 236, 237, 239
inositol phosphates, 6
whole raw seed studies, 13

O
Oats
inositol phosphate degradation during processing, 21
zinc absorption influence, 33
Oils, ultrasonic analysis
particle sizing, 150
spoilage correlations, 153
viscosity, 147
Ω-gliadins, 222
structural studies, 278
Oncorhynchus mykiss (rainbow trout)
culture, ozone water treatment, 181, 190
Onions
inositol phosphates content, 23
ozone treatment, 198
Oranges, ozone/carbon dioxide/argon mixed gas treatment, 201
Ozone, 167–208
analytical methods, 204–205
atmospheric, 170–171
biological oxygen demand (BOD) of water, 170
chemical oxygen demand (COD) of water, 170
chemistry/physics, 170–177
cycloaddition reactions, 175
decomposition, 176–177
catalysis by inorganic compounds, 177
free radical species generation, 176
inhibition, 177
initiation, 177
promotion, 177
electrophilic reactions, 175
microbial inactivation, 170
biofilms, 187, 198–199
effect of oxygen demand of medium, 181, 182
food characteristics influencing efficacy, 190, 191
food sanitizing applications, 189
kinetics, 187–188
mechanisms, 187–188
pulsed electric field combined treatment, 188, 203–204
relative humidity/microbial cell hydration, 179
residual ozone values, 180-181
spores, 186, 187
targeted microorganisms, 186–187
nucleophilic reactions, 175
oxidation–reduction potential, 171
pesticide residue reduction, 170, 189, 199
physical properties, 171
processing equipment interactions, 183–185
materials compatibility, 184
metals, 184–185
plastics, 185
rubber, 185
production methods, 204
reactivity, 175–177
inorganic compounds oxidation, 177
molecular ozone, 175
solubility, 172–173
stability, 173–175
structure, 171
toxicity, 206–207
transfer from gas to liquid phase (contacting systems), 182–183
treatment chambers, 182
treatment medium, 178–181
oxygen demand, 170, 181, 182
relative humidity, 178–180
residual ozone, 180–181
temperature, 178
workplace exposure thresholds, 207–208

Ozone sanitization
combination treatments, 200–204
carbon dioxide, 201–202
chlorine, 201
heat, 202
hydrogen peroxide, 200–201
pulsed electric field, 188, 203–204
ultraviolet radiation, 202–203
dry foods/ingredients, 198
equipment treatment, 192
filtration of treatment water, 183
fish, 197–198
food processing applications, 186–199
chlorine treatment alternative, 188–189
environmental contaminants reduction, 192
food characteristics influencing ozone-demand, 190–191
food surface structure, 190–191
limitations, 206
safety issues, 207
fruit, 190, 191, 192, 194–195
meat, 186, 189, 190, 191, 192, 193–194
metal contaminants removal from drinking water, 177
packaging materials, 192, 198–199
pesticide residue reduction, 170, 189, 199
poultry carcasses, 183, 190, 192, 193–194, 201
raw products/ingredients, 191–192
regulatory status, 206
shelf-life extension, 193
storage applications (ozone gas), 178
surface decontamination, 178
food contact surfaces, 198–199
vegetables, 190, 191, 194, 195–196

P
Packaging materials, ozone treatment, 192, 198–199
Packeria candidissima, 77–78
Paramecium phytase, 11
Parenteral fat emulsions, ultrasonic particle sizing, 150
Parsnips, inositol phosphates content, 23
Patternson's Curse (Echium plantagineum), 72
Pea flour, inositol phosphate degradation during processing, 21
Peanuts
aflatoxins, ozone treatment reduction, 198
phytate content, 13
Pedicularis (betony), 77
Petroselinum sativum, 76
Phaseolus vulgaris L., iron availability of low-phytic acid varieties, 34–35
Phonons, 103
Phosphatases, 24
Phospholipase C, 27
Phytases, 8–9
activation in seeds/grains before cooking, 9
animal digestive tract, 24
cell/tissue activity, 9–10
animal cell lines, 24
classification, 11
phytate degradation during food processing, 16, 20, 21
purified enzymes activity, 10
Phytate see Inositol hexakisphosphate
Phytophthora parasitica, 181
Pipe fouling, ultrasonic detection, 153
Plant pyrrolizidine alkaloid sources, 62–65
Plastic pouches, ultrasonic leak detection, 154
Polymer hydration, ultrasonic analysis, 151–152
Potatoes, inositol phosphate content, 23
Poultry carcasses, ozone treatment, 190, 192, 193–194
hydrogen peroxide combined treatment, 201
prefiltration of treatment water, 183
residual ozone values, 180
Protein hydration, ultrasonic analysis, 151–152
Pseudomonas aeruginosa, 180
Pseudomonas fluorescens, 180, 188, 199
Pseudomonas putida, 180
PTEN, 29
Pulsed electric field/ozone combined sanitation procedures, 188, 203–204
Pulsed mode ultrasonic measurements, 123–124
Punctaneine, 77
Pyrrolizidine alkaloids, 61–93
carcinogenic potential, 62, 66, 78, 81
chemical structure, 65–66, 67, 71
consumer protection measures, 69, 73–74, 91–93
food contamination, 66, 68–73
cereal grains contaminated with weeds, 66, 68–79, 91
eggs, 73, 91
honey, 72, 91
meat, 73
milk, 70, 72, 91
preventive measures, 91
herbal medicine sources, 66, 68, 73–78
adverse health effects awareness, 91, 93
metabolism, 81–83, 84–85, 86–87
dehydrogenation, 82
detoxification, 83
N-oxidation, 82–83
plant sources, 62–65
toxicity, 62, 78–81
acute, 78
cardiopulmonary, 80–81
diagnostic problems, 81
DNA cross-links formation, 83, 88–90
DNA–protein cross-links, 90, 92
hepatic veno-occlusive disease, 69–70, 72, 75–76, 77, 79–80, 91
hepatotoxic synergism with copper, 79
human poisoning incidents, 62, 68, 73, 75–77
mechanism, 83, 88–91
megalocytosis, 88, 89
progressive nature, 79–80
species differences in susceptibility, 78–79
structural determinants, 65
transplacental transmission, 76

Q
Quinoa floor, inositol phosphates
degradation during processing, 22

R
Rainbow trout (Oncorhynchus mykiss)
culture, ozone water treatment, 181, 190
Raleigh waves, 104–105
Retronecine, 66, 67
Retrorsine, 78, 79, 80, 88
Rice
Aspergillus fumigatus phytase
transgenic expression, 34
phytate (inositol hexakisphosphate) content, 12, 13, 16
reduced phytate gene mutations, 34
Riddelliine, 80, 88
Rockfish, ozone treatment, 197
Russian comfrey (Symphytum uplandicum), 74–75
Rye
phytase, 9, 21
phytate (inositol hexakisphosphate), 9
degradation during processing, 21

S
Saccharomyces cerevisiae phytase, 11
Salad dressings, ultrasonic particle sizing, 150
Salmon, ozone treatment, 192
Salmonella enterica ser. Enteritidis, 193, 194
Salmonella spp., 169, 183, 193, 194
Salmonella typhimurium, 180, 203
Salvation Jane (Echium plantagineum), 72
Sanitization methods
  ozone applications see Ozone
  sanitation
  requirements for food safety, 169–170
Scanning acoustic microscopy, 156
Schizosaccharomyces pombe, phytate
  (inositol hexakisphosphate)
  synthetic pathway, 7
Scyllo-inositol, 37
Seeds, inositol phosphates content, 12–15
Selectins, 30
Selenium absorption, phytate (inositol hexakisphosphate) effects, 32, 35
Senecio, 80
  contamination of staple foods, 68, 69–70
  prevention, 91
Senecio burchelli, 69
Senecio disease, 69
Senecio ilicifolius, 69
Senecio jacobaea (tansy ragwort), 70, 72
Senecio longilobus, 77
Senecio riddellii, 65
Senecio triangularis, 77
Senecionine, 66, 67, 72, 76, 77, 78, 80, 88
  metabolism, 82, 84–85
Seneciphylline, 71, 72, 76, 78, 80, 88
Senkirkine, 66, 67
Shelf-life extension, ozone treatment, 193
Shrimp meat, ozone treatment, 197–198
  residual ozone values, 180
Sicco near isogenic line, 251–252
  dough properties, 259–261
Sockeye salmon, ozone treatment, 197
Solid fat content, ultrasonic sensing
  isothermal scanning, 139–140
  temperature scanning, 141
Sorghum flour, phytate content, 16
Sour dough breads, phytate content, 20
Soybean
  inositol phosphates
    calcium absorption influence, 35
    degradation during malting, 22
    phytate (inositol hexakisphosphate), 12
    reduced content gene mutations, 34
    synthetic pathway, 8
Spirodela polyrhiza, 8, 23
Staphylococcus aureus, 180
Strawberries, ozone treatment, 192
Sunflower seeds, phytate content, 13
Supinine, 73
Symphytine, 75
Symphytum (comfrey), 74–76, 91
Symphytum uplandicum (Russian comfrey),
  74–75
Symviridine, 75
Synaptotagmin, 30
T
Tansy ragwort (Senecio jacobaea), 70, 72
Temperature, ultrasonic sensing, 132–133
Texture, ultrasonic sensing, 142–148
  viscosity measurements, 147–148, 149
Thermal degradation, inositol phosphates,
  16
Thermal sanitization, ozone sequential procedures, 202
Tomato sauce, ultrasonic Doppler velocimetry, 148, 149
Tomatoes
  ozone treatment, 191
  washing water-related salmonellosis
  outbreaks, 169
Transgenic wheat
  breadmaking quality differences, 262–265
  transformation system, 253–254
Trichodesmine, 67, 78, 80
Triglycerides, ultrasonic phase transition monitoring, 141
Triticum dicoccoides, 253
Triticum monococcum ssp. boeoticum, 226
Triticum monococcum ssp. monococcum, 226
Triticum timopheevi, 228
Triticum tsudchii, 228
Triticum turdium ssp. dicoccoides, 226
Triticum urartu, 226
Tritordeum
  transformation, 255–256
  transgenic line breadmaking qualities, 263
Turnip, inositol phosphates content, 23
Tussilago farfara (coltsfoot; coughwort), 76, 91
U
Ultrasonic composition sensors, 133–139
  application to animal carcasses, 138–139
Ultrasonic flow meters, 130–132
doppler flowmeters, 131–132
transit-time mode, 130–131
vortex shedding, 132
Ultrasonic imaging, 153–157
carcass grading, 154
high-resolution, 156–157
limitations, 155
noncontact methodology, 155
scanning acoustic microscopy, 156
Ultrasonic measurement systems, 121–129
air–transducer impedance barrier, 124
digitizer, 122–123
guided wave sensors, 128
Love wave sensors, 128–129
noncontact measurements, 124–126
pulsed modes, 123–124
pathlength measurement, 124
reflectance methods, 127–128
resonance methods, 126–127
resonance cell losses (Q-factor), 127
signal generator, 122
ultrasonic transducers, 122
Ultrasonic phase transition monitoring, 139–142
isothermal scanning, 139–140
temperature scanning, 140–142
Ultrasonic sensors, 101–158
advantages/disadvantages, 157–158
applications, 129–157
composition, 133–139
dispersed systems, 148, 150–151
fluid flow, 130–132
imaging, 153–157
level sensors, 129–130
phase transitions, 139–142
polymeric systems, 151–153
rheological properties (texture), 142–148
temperature, 132–133
viscosity measurements, 147–148
nondestructive testing, 104
theoretical aspects, 103–121
Ultrasonic thermometry, 132–133
Ultrasonic transducers, 122
Ultrasonic wave propagation, 103–104
attenuation, 105, 109–111
coefficient, 105–106
overestimation, 120
relaxation, 111
diffraction, 120–121
homogeneous materials, 105–111
adiabatic elastic modulus, 106
properties of materials, 106, 107–108
inhomogeneous media, 112–117
emulsions, 112, 114–116
floculated emulsions, 116–117
multiple scattering theory, 113–116
particle sizing, 115–116
scattering interactions, 112–113
interfaces, 117–120
acoustic impedance, 117–118
reflection coefficient, 118, 119
refractive index, 118–119
transmission coefficient, 118, 119
longitudinal (compression) waves, 104,
105, 106, 109
critical angle interactions at
boundaries, 119–120
near-field/far-field regions, 121
shear (transverse) waves, 104, 105,
109
surface waves, 104–105
Love, 104
Raleigh, 104–105
velocity, 105, 106, 109
adiabatic compressibility calculation,
151
gases, 106, 109
liquids, 109
solids, 109
wave characteristics, 103–105
Ultraviolet radiation, ozone combined
sanitization procedures, 202–203
Uplandicine, 72
V
Vanadium, inositol phosphate interactions,
36
Vegetables
chlorine treatment, 188
in-plant contamination, 194
inositol phosphates, 22–23
ozone treatment, 190, 191, 192, 194,
195–196
pesticide residues reduction, 199
sanitization methods, 170
texture, ultrasonic sensing, 142
Vibrio cholerae, 180
Vibrio parahaemolyticus, 180
Viscosity, ultrasonic measurement, 147–148, 149
von Kármán vortex street, 132
Vortex shedding, 132

W
Wheat flour
calcium bioavailability, 35
iron absorption influence, 33–34
ozone treatment, 192
zinc absorption influence, 33

Whey protein, ultrasonic analysis, 152, 153
Wine, ultrasonic alcohol determination, 138
Winery equipment, ozone treatment, 192

Y
Yogurt, ultrasonic sol–gel transition monitoring, 147

Z
Zinc absorption, inositol phosphates influence, 32, 33, 35, 36, 42
Zygosaccharomyces bailii, 197