

TRENDS IN  
**FOOD SCIENCE  
& TECHNOLOGY**

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May 1991

US Cooperative  
Extension Service

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Plant cultures for  
food ingredients

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Enzyme engineering

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Carotenoids: a new  
RDA?

Molecular modelling in  
food research



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# Modelling food systems

Scientists have long relied upon the use of models, based on existing hypotheses or knowledge, to study the behaviour of chemical and biological systems. Notable examples include the tetrahedral model of the carbon atom proposed by van't Hoff and Le Bel, Kékulé's model of the benzene molecule, and the models of Dreiding, which represented bonds between atoms as sticks connected together at the appropriate angles, each junction representing an atom.

From the late 1960s, computer-assisted molecular modelling extended and developed such early models. However, it was not until the mid-to-late 1980s, when the present generation of powerful graphics work stations appeared on the market, that the technology became refined enough, user-friendly enough and cheap enough to facilitate widespread use. Despite the complexity of food systems and the difficulty of obtaining crystal structures for many food proteins, molecular modelling techniques are now finding applications in food research (see 'Molecular modeling in food research: technology and techniques', by Thomas F. Kumosinski, Eleanor M. Brown and Harold M. Farrell, Jr, and 'Enzyme engineering', by Richard W. Pickersgill and Peter W. Goodenough in this issue).

In its simplest form, molecular modelling can be used as an alternative to the familiar plastic kits to display virtual images of molecules using, for example, Dreiding, ball-and-stick or space-filling (Corey-Pauling-Koltun; CPK) models. More sophisticated displays can present molecules in three dimensions (e.g. using anaglyphs – stereoscopic images – together with the appropriate viewing spectacles, or using the 'depth cueing' technique, in which light intensity is used to create an impression of depth on a two-dimensional screen); some images may be rotated in real time on the more powerful systems. Visualization of other aspects of molecular structure and function is also possible: the solvent-accessible surface, van der Waals radii and, if atomic partial charge data are available, molecular electrostatic potential energy surfaces may be modelled.

A major advantage of modern molecular modelling techniques is that models can be created and modified quickly, and their conformational and electrostatic properties can be studied without having to synthesize the molecules. Such approaches may save an enormous amount of time in the screening of molecules for a particular activity or property. Thus, the usefulness of molecular modelling has been enhanced by the simultaneous development of methods for the study of quantitative structure-activity relationships (QSAR) and quantitative structure-property relationships (QSPR).

However, despite the sophistication of computer modelling methods, scientists should be aware that the computer-generated structures are still only models and, consequently, the molecular data derived may not correspond to the real system. Some problems arise from the experimental methods used to produce the data that are used as a basis for building the molecular models; for example, the properties of a molecule in solution may differ from those predicted by the solid-state crystal

structure, as determined by X-ray diffraction analysis. Fortunately, Fourier transform infrared spectroscopy and two-dimensional nuclear magnetic resonance spectroscopy coupled with so-called distance geometry methods have helped to advance understanding of the solution structures of molecules.

A second set of problems associated with computer modelling arises from the need to restrict the model to a level of complexity that can be mathematically represented by the computer system; computers are not yet powerful enough to model every property and interaction of a molecule economically or on a practical timescale. Thus, the frequently employed technique of minimizing the conformational potential energy of molecules is usually conducted 'in vacuum'; the inclusion of solvent molecules is limited to very small molecules or fragments of large molecules and, even then, necessitates the use of powerful computers. Furthermore, as pointed out by Kumosinski *et al.*, the algorithms used to calculate molecular properties are usually semi-empirical representations of quantum mechanics functions or, occasionally, are based on the fully empirical force-field methods. There is much support for the use of *ab initio* molecular orbital methods based on quantum mechanics, because the calculations are more scientifically correct. However, *ab initio* calculations require large amounts of time on powerful computers. In addition, the semi-empirical methods are continually being refined as the experimental data sets used as the basis for such calculations are improved. Thus, considering that the relative simplicity of semi-empirical calculations may facilitate the inclusion of solvent molecules and other interactions, such models may compare favourably with the *ab initio* approach.

How might molecular modelling be useful to food research? Clearly, as mentioned by Kumosinski *et al.* and by Pickersgill and Goodenough, one possible application is the study of food proteins and peptides, and their modification to produce food ingredients with tailor-made properties. Many such studies of food macromolecules are still at the basic research stage. However, an area of food research that has been using molecular modelling techniques for many years is the study of sweet taste and the development of new sweeteners.

Problems important to the study of food systems remain to be addressed. For example, it is important to model the effects on molecular properties of hydration, co-solutes and temperature, as soon as the computer technology is powerful enough to enable such calculations. As the new models of food systems become refined, it will be possible to design new foods with a degree of specificity that will justify the expense of the initial 'basic' research.

# Food science and Cooperative Extension: a view of the past and a vision for the 21st century

Donald W. Schaffner

The next century will offer challenges and opportunities to all of humanity as the rate of technological change increases. Since all of humanity must eat to survive, food will continue to play a central role in our lives, even as the technological changes take place. Unique challenges concerning the food supply will face us in the next century. The Cooperative Extension Service of the US Department of Agriculture can help to bring together food scientists, food producers and consumers to face these challenges.

The challenges facing the food scientist in the 21st century can be stated in the form of several questions, which will be addressed in this feature.

- How can we better understand the concerns of consumers, continue to educate them, and expand their role in assuring a safe food supply?
- How can we continue to interest clever students in careers in food science, and ensure that all students are scientifically literate?
- How can we ensure continued innovation and progress in food technology, especially in small companies?

The Cooperative Extension Service, part of the US Department of Agriculture (USDA), provides the means by which each of these questions can be answered. The Service, which just celebrated its 75th anniversary, has a long and colorful history. The motto for the 75th Anniversary celebration was 'Practically remarkable . . . remarkably practical'; these words accurately describe the history of Cooperative Extension.

Cooperative Extension is one part of the tripartite mission of the US land-grant colleges. These institutions were established by a bill introduced on 14 December

1857 by Representative Justin R. Morrill of Vermont. The bill gave a grant of lands to the states to establish colleges to teach agriculture and the 'mechanical arts'. The Morrill Act gave each state 30 000 acres of land for each member it had in the House of Representatives or in the Senate. In many cases, the land was not located within the state, and was sold for less than a dollar an acre. The proceeds from the sale of the land were used to establish the agricultural and mechanical colleges that Morrill had envisioned. The original Morrill Act provided a lump sum for the establishment of the colleges, but it wasn't until the Second Morrill Act was passed in 1890 that annual funding for these colleges was established.

In the meantime, the colleges had another problem. While they were founded to teach agriculture, only a very limited scientific understanding of the principles of agriculture existed. A bill sponsored by W.H. Hatch of Missouri and J.Z. George of Mississippi was introduced in 1882 to remedy the problem. The Hatch Act was finally made law in 1887, putting into place the second part of the land-grant mission: research.

It was now possible to conduct agricultural research and to teach it at the colleges, but a problem still remained. The existing system disseminated information slowly, and could not convince many farmers with a strong distrust of 'book learning' that the new methods were better. A method for effectively disseminating information to those who needed it was lacking.

At this time, the USDA was already distributing some printed information to farmers, largely as a development of its activities as a division of the Patent Office. In 1898, a unique approach was tried in Louisiana. The USDA hired Seaman A. Knapp, an agricultural teacher, to promote agriculture in the South. Knapp identified in each township one farmer willing to let his farm be used as a demonstration farm for the area. Thus, Knapp applied his philosophy: 'What a man hears, he may doubt; what he sees, he may possibly doubt; but what he does, he cannot doubt.'

Other activities were underway that would also contribute to the formation of Cooperative Extension. Boys' and girls' clubs were founded in many states to motivate young people and to educate them about farming. These clubs sponsored tomato-, corn- or flower-growing contests, with prizes for the best entries. Home demonstration clubs educated farm women about smokeless cooking, and about canning and marketing farm produce. The clubs provided a means of increasing income as well as a chance to socialize.

President Roosevelt's Country Life Commission proposed that these three 'outreach' activities (agriculture, youth clubs and home demonstration clubs) be nationalized, and linked to the state colleges of agriculture. Bills based on these recommendations were introduced in 1909 and 1910 to the House of Representatives and to the Senate, but were unsuccessful; additional bills in 1911, 1912 and 1913 were also unsuccessful. Finally, in 1913, a bill sponsored by Asbury F. Lever of South Carolina and Hoke Smith of Georgia was introduced that provided for a cooperative system of education, with participation by

the USDA and the state agricultural colleges. This bill was signed into law in 1914 by President Woodrow Wilson, who said it was 'one of the most significant and far-reaching measures for the education of adults ever adopted by the government'. Thus, President Wilson established the third component of the land grant college system: the Cooperative Extension Service.

The Extension Service has a rather unique structure, which should be explained. Communication is facilitated by national program leaders based at the USDA in Washington, DC. Program leaders monitor activities in each state, and promote contact between states. The next level within Cooperative Extension is composed of state specialists, who lead and coordinate activities within a particular state. These specialists may also participate in the other two missions of the land grant colleges: research and teaching.

Finally, the 'front line' of the Extension Service is the county office. Here, several different types of professionals lead multidisciplinary programs designed to assist their clients. Personnel include agricultural advisers who assist farmers, home economists who assist consumers, and '4-H' (youth) agents. An agricultural agent may assist farmers with many different problems, such as those of vegetable production, the growing of grain, or the raising of dairy cattle. A home economist may conduct financial planning workshops and answer telephone inquiries about food safety; a 4-H agent may educate inner-city youth about the dangers of drugs, or judge a vegetable-growing contest at a county fair.

Initially, food science and technology was not covered by the agricultural colleges; teaching, research, and extension services focused on the production side of agriculture. However, as faculty members developed expertise and an understanding of areas other than production agriculture, food science departments were formed from existing departments such as animal science, dairy science and horticulture. Some food science departments were also formed without ties to existing departments; the Department of Food Science at Rutgers University, with its 'discipline' orientation of dividing food science into food biology, food chemistry and food engineering is an example of such a department.

With a clear understanding of the origins of the land grant colleges and of the Cooperative Extension Service, we may examine the three questions posed at the beginning of the article.

### **How can we better understand the concerns of consumers, continue to educate them, and expand their role in assuring a safe food supply?**

Consumers are not confident in the safety of the food supply. The attention devoted to the pesticide 'Alar' two years ago showed that consumer confidence can be undermined by a carefully orchestrated media campaign. The problem is compounded by the fact that many more serious food safety issues (such as microbiological food poisoning) are often overlooked. The success of innovations such as chilled foods depends upon the proper handling of these foods by consumers.

County home economists have been teaching food safety for many years, just as they taught consumers how to can foods at home. Furthermore, a great deal of trust exists between clients and county agents because of the years of mutually beneficial interactions. If home economists listen to consumers who are worried about food safety, and if they understand the concerns of the consumers, successful education programs can be developed. A specialist who knows about food safety and a home economist who knows the needs of consumers should interact synergistically to communicate the appropriate information to consumers.

### **How can we continue to interest clever students in careers in food science, and ensure that all students are scientifically literate?**

It has been said that the USA is facing a crisis of scientific literacy, in a generation that depends on technology, but cannot understand it. The problem is even beginning to manifest itself in undergraduate food science programs. A lack of interest in science, compounded by a shrinking population may undermine our scientific educational infrastructure. The county 4-H youth agent is in a position to expose young people to food science at an early age. Moreover, if a youth agent who knows the client base, and a specialist who understands food science can develop an educational program together, the result could be the formation of a core of future food scientists. Demonstrations of a few simple experiments that can be done in the kitchen could be enough to create a pool of talented food scientists for the future.

### **How can we ensure continued innovation and progress in food technology, especially in small companies?**

Innovation often has its roots in small companies that must solve problems in original, creative ways in order to succeed. Such companies may eventually grow and become parts of larger companies. However, the problems facing a small food processing plant or an entrepreneur with an idea for a new food product often seem insurmountable. A small food company may not have research and development staff, or it may rely on someone who also has other responsibilities to fulfill this role. An entrepreneur may develop a promising new product at home in the kitchen, but be unaware of how to turn the idea into a safe, marketable product. A state specialist or a specialist and an agent working together can share their accumulated knowledge with the entrepreneur by suggesting a solution, or by pointing out potential pitfalls. If more information is needed, the specialist can draw on the resources of other food scientists, colleagues in other states, and independent consultants.

By informing and assisting new or small food processing companies, the Extension Service can help to promote the continued viability and profitability of the food industry. Clearly, in addressing the challenges discussed above, programs such as the Cooperative Extension Service of the USDA and the US agricultural colleges can be a tremendous support for food science in the next century.

# Molecular modeling in food research: technology and techniques

Thomas F. Kumosinski,  
Eleanor M. Brown and  
Harold M. Farrell, Jr

The development of structure–function relationships is essential to the effective use of biotechnology for solving problems in food science and technology. The introduction of more powerful computers, together with the increased availability of efficient molecular modeling software will enable the study of such relationships. In this review, we present the most important techniques used in the modeling of small molecules, proteins, nucleic acids and polysaccharides. A brief description of the application of experimental techniques for structure determination, such as X-ray crystallography, two-dimensional nuclear magnetic resonance spectroscopy, circular dichroism spectroscopy and Fourier transform infrared spectroscopy is provided, with particular emphasis on their relevance to molecular modeling. Various techniques for determining the minimum conformational potential energy, ranging from rigorous molecular orbital methods based on quantum mechanics to approximate molecular mechanics methods based on force-field calculations, are described; the selection of appropriate methods to study particular sizes and types of molecules and molecular motions is also presented.

Biotechnology promises the development, by the new techniques for the genetic engineering of proteins, of products with tailor-made functionalities for food manufacture, and the creation of new co-solutes that may control functionality. However, the historic problem of

Mention of brand or firm names does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

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developing quantitative structure–function relationships still exists. Without knowledge of such relationships, the new techniques are limited in potential and have a low probability of success.

The caseins of bovine milk and their naturally occurring genetic variants provide an illustration of qualitative correlations between protein primary structure and functionality in food systems. For example, cheeses made from milks containing  $\alpha_{S1}$ -casein A have a softer texture and body than those made from the more frequently occurring  $\alpha_{S1}$ -casein B variant; milks containing the  $\alpha_{S1}$ -casein A variant are also more resistant to calcium-induced coagulation. The  $\alpha_{S1}$ -casein A variant is the result of the deletion of 13 amino acids (residues 14–26) from the B variant<sup>1</sup>. In  $\beta$ - and  $\kappa$ -caseins, single-site mutations change the chymosin-induced clotting time, the initial step in cheese manufacture<sup>1</sup>. However, the changes in secondary, tertiary and quaternary structure resulting from such mutations have not been obvious. Thus, the mechanism of the functionality changes described above is poorly understood, and the success rate of future deliberately induced mutations cannot be predicted.

In recent years, the development of molecular modeling as a technique for developing or refining three-dimensional molecular structures has resulted in a methodology capable of suggesting a molecular basis for structure–function relationships. At present, the behavior of food proteins, preservatives, emulsifiers and stabilizers can be modeled in a food system. Studies of the structure–function relationships of new peptides, carbohydrates, polysaccharides and small molecules can be used to test their potential effectiveness.

The purpose of this report is to provide a synopsis of the various techniques in molecular modeling and to guide interested food scientists in acquiring the most useful technologies, both software and hardware, for solving their specific problems. A future report in this journal will deal with specific applications of various techniques for solving structure–function relationships.

## Techniques for the determination of three-dimensional structure

The traditional method for determining the three-dimensional structures of proteins and smaller molecules is X-ray crystallography. The methodology enables the elucidation of structure with a high degree of precision<sup>2</sup>. In fact, the structure of a small molecule can be determined from the diffraction pattern of its crystals in a relatively short time using so-called ‘direct methods’ (see Glossary). However, for macromolecules (e.g. proteins and nucleic acids), the solution is not straightforward, and it is generally necessary to prepare several different heavy-atom isomorphous derivatives. The diffraction patterns for the crystal with and without the heavy metal are then obtained and the results are analysed using Patterson synthesis. The limitations of X-ray crystallography for the determination of structure–function relationships in proteins include the requirements that the sample be crystalline and that a suitable heavy metal be incorporated without distorting the crystal.

Unfortunately, many proteins and other food molecules cannot be crystallized at present and, of course, the functional form of the molecule usually exists in the solution, gel or sol state – not in the form of a crystal.

In contrast to X-ray crystallography, two-dimensional nuclear magnetic resonance (NMR) spectroscopy can measure the structure of a molecule in solution<sup>3</sup>. The methodology is not as precise as X-ray crystallography, is time-consuming and is limited to the study of proteins with less than 100 amino acid residues. Two types of NMR experiment are used: correlation spectroscopy (COSY), which uses the spin–spin coupling between protons to establish band frequencies for specific amino acid residues, and nuclear Overhauser enhancement spectroscopy (NOESY), which measures the ‘through space’ dipole–dipole coupling between protons. At a given band frequency, the dipole–dipole coupling constants measured by NOESY are directly proportional to the correlation times of the magnetically coupled protons, and are inversely proportional to the cube of the distance between them. Thus, the NOESY data provide a measure of the relative motion of the protons and of the ‘through space’ distances between them. The calculated ‘through space’ distances can be used to derive a plausible three-dimensional static structure for a small molecule or protein, provided that there is a large number of well-resolved NMR bands. The larger the number of bands, the greater the precision and accuracy of the resulting structure. Thus, the technique requires a high-field NMR spectrometer; usually a frequency of 500 MHz is needed for acceptable proton resolution and to obtain two-dimensional spectra. In addition, higher molecular weight proteins have longer correlation times, larger nuclear Overhauser effects and larger band widths, all of which decrease the resolution while increasing the number of bands. Hence, the current size limit is 100 amino acid residues.

### Techniques for the determination of secondary structure

The most widely used technique for the estimation of the secondary structure of a protein is circular dichroism spectroscopy<sup>4</sup>. The technique measures the dependence on wavelength of ellipticity (the difference in the absorbance of left and right circularly polarized light) of the optically active peptide bonds in the far ultraviolet region, mainly 230–185 nm. Three bands with either positive or negative maximum ellipticities are produced for each of the standard conformational states ( $\alpha$ -helix,  $\beta$ -pleated sheet and random coil); one is produced for the  $n \rightarrow \pi^*$  transition (energy absorption by a non-bonding electron), and one each for the parallel and perpendicular  $\pi \rightarrow \pi^*$  (double-bond absorption) electronic transitions of the electrons of each peptide bond. The spectrum observed for residues in an  $\alpha$ -helix conformation is much more intense than those for other conformations; thus, the method is most reliable for quantifying the content of  $\alpha$ -helix. The need to have optically clean solutions (any components that scatter light will affect the results) and very accurate determinations of protein concentration

### Glossary

**Direct methods:** Statistical methods for determining the phases of the diffraction beams in an X-ray diffraction experiment.

**Isomorphous derivatives:** Another approach to determining the phases in X-ray diffraction experiments that involves replacing an atom in the molecule by a heavy atom, without appreciably distorting the crystal structure.

**Patterson synthesis:** Yet another approach to the determination of phases in X-ray diffraction experiments. This technique may be used in conjunction with the isomorphous replacement method, and involves the use of the mathematical technique of Fourier synthesis to calculate the electron densities from the diffraction beam intensities.

**Nuclear Overhauser effect (NOE):** The saturation of the resonance signal that occurs when nuclei are irradiated to the extent that the nuclear energy levels become equally populated. Observation of NOEs can give information about internuclear distances.

**Self-consistent field (SCF) orbitals:** This refers to the mathematical approach used to calculate the orbitals; initially, a set of orbitals is assumed and the electron–electron repulsion is calculated. The energy obtained is then used to calculate a new set of orbitals. The process is repeated until convergence and self-consistency is achieved for the orbitals.

complicate the method. However, an advantage of the method is the need for only small volumes of dilute aqueous protein solutions.

On the other hand, Fourier transform infrared (FTIR) spectroscopy experiments can be applied to samples in any state (solid, solution, gel, sol, etc.). However, protein concentrations must be high (20–50 mg/ml), and D<sub>2</sub>O rather than H<sub>2</sub>O is used for solution-phase experiments. The resolution of the amide I band, which arises from the peptide bonds, can be increased by the use of Fourier transform deconvolution techniques<sup>5</sup>. Individual frequencies within the broad amide I band can be calculated. Since a large signal-to-noise ratio is obtained using FTIR, various mathematical techniques can be used to enhance the spectra. The resulting deconvoluted and enhanced spectra can then be fitted to a sum of individual Gaussian peaks using nonlinear regression analysis to determine the relative contributions of individual bands to the observed broad amide I band. The frequencies of the individual bands can, in turn, be correlated with secondary structural assignments using the theoretical calculations of Krimm and Bandekar<sup>6</sup> in combination with the results of FTIR spectra obtained for proteins for which three-dimensional X-ray crystal structures are available<sup>5</sup>. Thus, the amounts of  $\beta$ -turn,  $\alpha$ -helix,  $\beta$ -sheet, extended strand or unordered structure can be estimated. Although this methodology is promising, its precision and accuracy for the determination of turn structures are still being assessed.

### Criteria for building molecular structures

Obtaining detailed three-dimensional images of food proteins by traditional methods may be impractical. Many food proteins do not readily form crystals or, at least, crystals suitable for high-resolution X-ray diffraction analysis. Moreover, facilities for structure determination by X-ray crystallography may not be available locally, and finding collaborators who are interested in a food protein may be difficult. However, the construction of a model based on previously determined physicochemical

and spectroscopic characteristics of the protein as well as on the predicted behavior of the amino acid sequence can provide a starting point for the exploration of structure–function relationships.

When deriving molecular models, it is imperative to use a library or a dictionary of geometric parameters to ensure that assigned bond lengths, bond angles, and van der Waals radii are compatible with those determined by X-ray crystallography. The resulting molecular models can then be compared with other experimentally determined structures. All of the major molecular modeling software packages have such libraries or dictionaries. Table 1 lists some of the software packages currently available for molecular modeling. The packages vary in terms of their facilities and possible applications, and the researcher should study such systems in detail to determine the most appropriate system for solving a particular problem that is compatible with the available hardware.

Each of the software packages listed in Table 1 contains libraries or dictionaries that are compatible with geometric parameters derived from X-ray crystallography. They are easily interfaced with the Brookhaven database of protein crystal structures<sup>7</sup> and the Cambridge crystallographic database (Cambridge Crystallographic Data Files, Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK), which contain most of the available X-ray crystal structures for proteins and small molecules, respectively. Each software package can calculate the potential energy field of a molecule, and incorporates one or more algorithms for minimizing conformational energy and, usually, an algorithm for modeling molecular dynamics. Models of small molecules, polysaccharides, polynucleotides and proteins can be ‘built’, their conformational energies minimized, and the structures compared with those determined experimentally. Finally, these packages interface easily with several programs, available from the Quantum Chemistry Program Exchange (QCPE) (Quantum Chemistry Program Exchange, Department of Chemistry, Indiana University, Bloomington, IN, USA), that calculate molecular parameters using quantum mechanics.

### Molecular orbital calculations

*Ab initio* self-consistent field (SCF) molecular orbital calculations (based on quantum theory) can be done routinely for small molecules (not proteins)<sup>8</sup>. Such calculations are generally based on some modification of

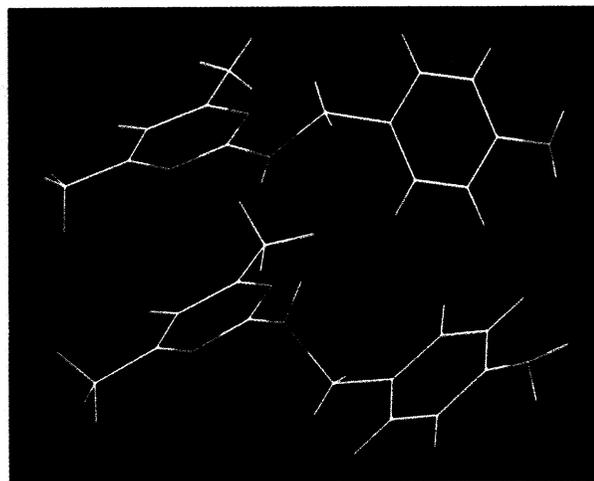


Fig. 1

The structure of sulfamethazine as determined by X-ray crystallography (above), compared with the energy-minimized structure (below). Carbon, white; hydrogen and nitrogen, blue; oxygen, red; sulfur, yellow.

the Hartree–Fock method, which approximates molecular orbitals as a linear combination of hydrogen-like, Slater-type or Gaussian orbitals; this linear combination of atomic orbitals is similar to an infinite series (Taylor series), which expresses a function as a sum of polynomial terms. The Hartree–Fock approximation is generally used in very time consuming, which makes *ab initio* calculations on large molecules impractical. *Ab initio* calculations are usually computed using one of the QCPE programs (‘Gaussian 80’ or ‘Gaussian 86’), and give molecular parameters such as atomic partial charges, dipole moments and quadrupole moments for moderately sized heteroatomic molecules of known crystal structure.

The most useful calculations for identifying the optimum geometry – the structure that has the lowest potential energy – are the semi-empirical techniques<sup>8</sup>. Unlike the entirely theoretically based *ab initio* methods, the semi-empirical methods are based on fitting experimental data to a series of models, and using the parameters obtained to predict the behavior of other molecules. Semi-empirical approaches enable the energy of a large molecule to be computed within a realistic time. Like the *ab initio* approach, these approaches usually start with some form of the general SCF molecular orbital theory; the semi-empirical approaches then make approximations to the quantum mechanics equations in an effort to reduce the number of terms to be computed.

Several semi-empirical programs are available from the QCPE, and differ only in their treatment of the cumbersome coulomb integrals (describing coulombic interactions between the two charge distributions) and exchange integrals; they include the ‘Complete neglect of differential overlap’ (CNDO), the ‘Intermediate neglect of differential overlap’ (INDO), the ‘Neglect of diatomic differential overlap’ (NDDO), the ‘Modified neglect of differential overlap’ (MNDO) and the ‘Modified intermediate neglect of differential overlap’ (MINDO and MINDO/3) packages. These programs are useful for finding the lowest conformational energy

Table 1. Software packages for molecular modeling

Package	Company
Biograph	Bio Design, Inc., Pasadena, CA, USA
Insight, Discover	Biosym Technologies, San Diego, CA, USA
SYBYL	Tripos Associates, Inc., St Louis, MO, USA
CHARMM, Quanta	Polygen, Waltham, MA, USA
Chem-X	Chemical Design Ltd, Oxford, UK
INTERCHEM	Interprobe Chemical Services, Glasgow, UK

state of a molecule by varying atomic distances and/or dihedral angles.

### Molecular force-field methods

Due to efficient algorithms, and the lower cost and increased availability of fast computers with large memories, calculations based on quantum mechanics have greatly increased the development of structure–function relationships for small molecules, in which the lowest potential energy and, hence, the most likely geometry are relatively easy to define. However, many problems of biological interest, such as the study of polynucleotide and protein conformation, can still be modeled only by using the most elementary empirical energy functions. Although such models are crude, the approach has been applied successfully to the study of hydrocarbons, oligonucleotides, peptides and amino acids during the past few years.

In the case of molecular mechanics or force-field methods, the molecule is represented by a collection of overlapping balls (the atoms, with given van der Waals radii) connected by springs (which mimic the vibrational character of the bonds). The atoms are assigned certain van der Waals attractive and repulsive forces as well as electrostatic forces, representing non-covalent interactions. Molecular mechanics methods use a combination of potential energy functions to optimize a structure. The three most important requirements for force-field calculations are an equation that calculates energy as a function of molecular geometry, the model parameters (a set of ‘best’ values for experimentally derived molecular properties), and an algorithm to calculate new atomic coordinates. Possibly, the most theoretically sound force-field package is ‘MM2’ by Allinger<sup>9</sup>, which is available from the QCPE. This package has been successfully used to determine the conformation of minimum potential energy of oligosaccharides and peptides, using an iterative approach<sup>10</sup>, but its use is limited to the study of molecules with less than 200 atoms. Other force-field methods, based on more empirical parameters derived from geometric and thermodynamic studies, have been successfully used for large molecules such as proteins, polynucleotides, synthetic polymers and polysaccharides. A recent review<sup>11</sup> examines several programs that use such methods (‘ECEPPS’<sup>12</sup>, ‘CHARMM’<sup>13</sup> and ‘AMBER’<sup>14</sup>). Although these software packages are quite sophisticated, they are still under development; at present, they are not able to handle hydrophobic interactions, and they do not take into account any interactions of the molecule with solvent, even in the form of a general term: molecules are studied in complete isolation (‘in vacuum’). The size of the molecule for which force-field calculations can be computed is limited by the speed and memory of the computer.

### Building sulfamethazine

Sulfamethazine is an important sulfa drug widely used as an antibiotic for treating mastitis in dairy cows<sup>15</sup>. A computer-generated image of its structure, based on the results of X-ray crystallography, is presented in Fig. 1. To

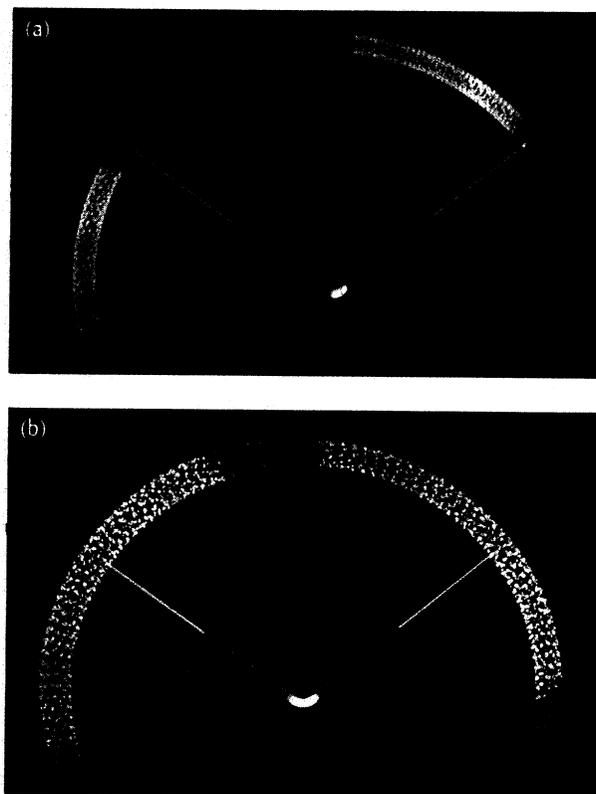


Fig. 2

Sweep graph of the dynamic motion of water, represented by a stick-model ‘V’, over 25 picoseconds: (a), at 300 K; (b), at 600 K.

The white area at the center of each image represents the location of the oxygen atom; arcs describe the locations of the hydrogen atoms during the 25-picosecond observation period. Color coding indicates the proportion of time spent by the hydrogen atoms in a particular location: red, high proportion of time; purple, low proportion of time.

test the validity of the molecular modeling algorithms, we used the ‘SYBYL’ molecular modeling package to build a model of sulfamethazine. The ‘Sketch module’ facility of the program enables the investigator to ‘draw’ the basic structure of the molecule from the constituent atoms, from fragments, or by modification of an existing structure. Bond lengths and angles can be adjusted to realistic values. To obtain the molecular structure of lowest potential energy (shown in Fig. 1 for comparison with the structure determined by X-ray crystallography), the energy of the sulfamethazine model was first minimized using a force-field model, which ignored electrostatic interactions. The energy of the resulting structure was further minimized using the MNDO program, to optimize the dihedral angles. The interatomic distances were already compatible with those determined by X-ray crystallography and, thus, were not optimized further. Comparison of the X-ray and computed structures indicates that the rotational angles for the C–N and N–P bonds of the two conformations differ. Since these bonds rotate freely when the molecule is in solution, the two structures may be considered to be in good agreement with each other; the energy difference between the two conformations is low. Atomic partial charges calculated for the X-ray structure using Gaussian 80 were in good agreement with those calculated using MNDO for the

energy-minimized structure. With such techniques it is now possible to examine newly synthesized drugs and other molecules to compare their molecular properties and to determine structure–activity relationships.

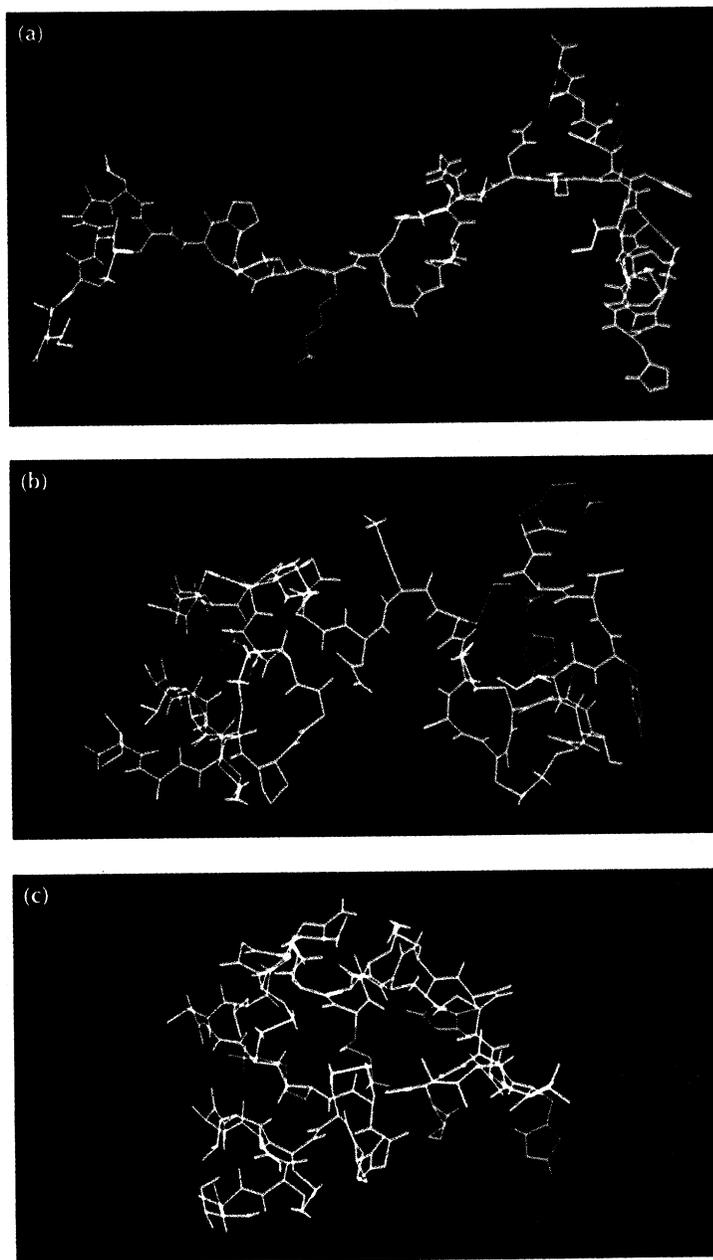


Fig. 3

Initial structure of nisin in a random-coil conformation. The so-called Ramachandran angles,  $\phi$  (the  $\alpha\text{C}_i$ –amino- $\text{N}_i$  angle) and  $\psi$  (the  $\alpha\text{C}_i$ –carbonyl- $\text{C}_i$  angle) of the peptide bonds were initially set at  $-60^\circ$  and  $60^\circ$ , respectively; the  $\omega$  angle between carbonyl- $\text{C}_i$  and amino- $\text{N}_{i+1}$  atoms was kept constant at  $180^\circ$ . (a), the open structure (blue, the backbone and neutral side chains; purple, basic groups except histidine; green, hydrophobic regions; yellow, sulfur). (b), the disulfide cross-linked energy-minimized structure of nisin after simulated annealing for 40 picoseconds at 350 K. Colors are as in part (a), except histidine residues are purple. (c), energy-minimized structures of nisin subjected to complete simulated annealing at temperatures between 1000 K and 300 K for a total of 100 picoseconds.

### Molecular dynamics and simulated annealing

The previous paragraph considered static structures only. However, the dynamic motion of molecules in solution contributes to their functionality. The molecular dynamics approach is a method of studying molecular conformation as a function of time. Each atom in the molecule is assigned a kinetic energy in the form of a velocity term, which can be related to local temperatures as well as to the average temperature of the system. Such calculations apply to molecules that are ‘in vacuum’ or in the presence of an appropriate number of molecules of a solvent such as water. In addition, the system being modeled can be studied at a constant temperature, volume or, in the future, pressure; in the case of pressure, a periodic boundary condition is defined to confine the system within a prescribed volume. For such molecular dynamics calculations, a force field describing the potential energy is combined with Newton’s second law of motion:

$$F_i = m_i a_i = m_i \frac{dv_i}{dt} = m_i \frac{d^2 x_i}{dt^2} = -\nabla_i E \quad (1)$$

where  $F_i$  is the force on atom  $i$ , which has mass  $m_i$ , velocity  $v_i$ , acceleration  $a_i$  and position  $x_i$ ;  $\nabla_i$  is the gradient or the derivative with respect to the position of atom  $i$ ;  $t$  is time;  $E$  is the potential energy of the molecule described by the force field, and is a function of the positions of all atoms in the molecule. Equation 1 is integrated over various time intervals using a numerical integration method. The time intervals must be small (usually one femtosecond) compared with the time period associated with the highest frequency of motion within the molecule (usually that of stretching a bond associated with a hydrogen atom). Numerical integration of Eqn 1 over one-femtosecond intervals for 100 picoseconds for a protein molecule of  $\geq 2000$  atoms requires a fast computer with a large memory. Such calculations can model the motions of molecules in solution. Time-dependent geometric parameters can also be modeled; for example, the distance from the center of movement for a group of atoms may be related to correlation times derived from NMR, electron paramagnetic resonance or fluorescence spectroscopy experiments.

Composite sweep graphs of the dynamics, over 25 picoseconds, of a water molecule at 300K or 600K are presented in Fig. 2 to illustrate the results of molecular dynamics calculations. The graphs illustrate a combination of the rotational and angle-bending motions of the water molecule, and clearly show the increase in molecular motion with increasing temperature.

Molecular dynamics calculations also facilitate the process known as simulated annealing<sup>16</sup>. It is well documented that local minima exist in the potential energy surface of molecular conformations. Hence, energy-minimization techniques can give rise to erroneous results when a calculation becomes trapped within such barriers, and may yield a structure that is not in the lowest energy state. Molecular dynamics calculations can overcome such computational barriers and permit calculations to continue until fluctuation about the lowest energy state occurs. However, long computation times that are

unrealistic may be needed. The simulated annealing method can help to overcome this problem. In simulated annealing, molecular dynamics calculations are performed for high temperatures (1000 K) until a constant energy is reached. The system is then 'cooled down' and further molecular dynamics calculations are performed at lower temperatures; each calculation is continued until the total energy is constant over time. When a structure corresponding to ambient temperature (or lower) is obtained, its energy can be minimized to yield the 'best' (lowest-energy) model. The simulated annealing calculation samples a large fraction of conformational space and minimizes the problem of local energy minima.

#### Building a nisin template structure

In the modeling of polypeptides and proteins, an extremely large number of possible conformational states exist, and a reliable methodology for choosing the initial structure based on experimental evidence is essential when attempting to build a protein structure. One approach is to use sequence-based predictions of secondary structure<sup>17</sup> in conjunction with circular dichroism or FTIR spectroscopy. Although the programs that predict the secondary structure of a protein from its amino acid sequence are based on data from X-ray diffraction analysis, the various programs yield different secondary structures. Where a consensus occurs among the different prediction methods, more reliance can be placed upon the estimates. In the absence of such consensus, the programs must be used in conjunction with global secondary structure experiments to find one or more plausible initial structures.

The modeling of nisin, a simple polypeptide of 34 residues with 5 cross-linked lanthionine bonds, is presented to demonstrate a possible approach to building a protein structure using molecular modeling techniques (Fig. 3). Nisin is an antibiotic peptide, which can be used in some dairy products and which may soon be used as a preservative in other food systems; it has recently been expressed in *Escherichia coli*<sup>18</sup>. For this demonstration, cysteine residues were substituted for the sulfur cross-linked lanthionines, and the dehydroalanine and dehydrobutyrine residues were replaced by alanine. The sequence-based secondary structure predictions were ambiguous, and suggested an unordered structure. The peptide has not yet been characterized spectroscopically; thus, no experimental secondary structure results were available. As any definite secondary structure prediction was unavailable, a pseudo-random structure with  $\phi = -60^\circ$  and  $\psi = 60^\circ$  was chosen for the initial structure. The open structure is presented in Fig. 3a. Because nisin is normally used under acidic conditions, all histidine residues were protonated, which yields a single positive charge. Disulfide bonds were added to the appropriate residues, and the structure was 'energy minimized' by simulated annealing at 350 K for 40 picoseconds (Fig. 3b). This structure was then subjected to simulated annealing calculations to the lowest total energy at each of 1000 K, 500 K, 400 K and 300 K for a total of 100 picoseconds (Fig. 3c). Comparison

of Figs 3a and 3c clearly shows how the simulated annealing process can overcome the problem of local energy minima.

Thus, a useful model is now available to determine surface residues that could be modified by genetic engineering to improve the properties of the molecule. The nisin structure (Fig. 3) appears to consist of two domains; it is suggested that one of the histidine residues may be in a small cleft and, therefore, have an altered  $pK_a$  reminiscent of some enzyme systems<sup>19</sup>. In subtilin<sup>18</sup>, a similar peptide in which histidine is replaced by asparagine, a difference in the small cleft is observed. Thus, molecular modeling of antibiotic agents, their probable receptor sites and the interaction of the two molecules can suggest means of developing new and improved antibiotics by the introduction of point mutations.

Undoubtedly, as the technology of molecular modeling develops, new applications in food research will be found. An extension of this review, to be published in a future issue of *Trends in Food Science & Technology*, will examine potential applications in more detail, in particular the applications of molecular modeling to the study of the structure-function relationships of food proteins.

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# The manufacture of food ingredients using plant cell and tissue cultures

Angela Stafford

Plant cell and tissue culture technology has reached the point where a variety of culture types can be critically assessed as potential sources of existing and novel food ingredients. Flavours and pigments have received most attention so far; cell suspensions that accumulate high levels of food pigments have been produced, and some may be suitable for commercial production. A number of large-scale processes that demonstrate the advanced state of plant cell culture technology have already been developed. It is likely that products for the food industry derived from plant cultures will be available for commercialization in the future.

Plants not only provide an essential source of nutrients, but also provide many ingredients that render foods palatable and visually attractive. Such ingredients, which are included in foodstuffs at low levels, are often of great commercial value, making them potential targets for novel production methods. Microorganisms have many traditional and new applications in foods, particularly in the production of flavours. However, so far, the use of plant cell and tissue cultures has had a low profile in the food industry, despite continuing interest in the technology by the pharmaceutical industry.

Ten years ago, plant cell and tissue cultures were viewed as potential alternative sources of virtually any plant-derived, high-value 'secondary product' (a chemical not essential for the day-to-day metabolism of the plant). Many of the targets were pharmaceuticals such as digoxin, quinine and morphine and, more recently, taxol. The potential advantages of plant culture systems are numerous: independence from environmental factors, independence of geographical location, uniformity, and controlled production in response to demand. Today, in the light of research conducted on an international scale, blind optimism has become educated realism, and there

is a much better awareness of the factors involved in determining the development and commercialization of a cell or tissue culture system for a particular product.

The critical factors governing the choice of a plant culture system in the place of field-grown crops are the potential product yield per annum and the production costs. The food industry is a potential user of ingredients, derived from plant cell and tissue cultures, with a variety of applications; flavours, pigments, sweeteners and enzymes are among those that will be considered here.

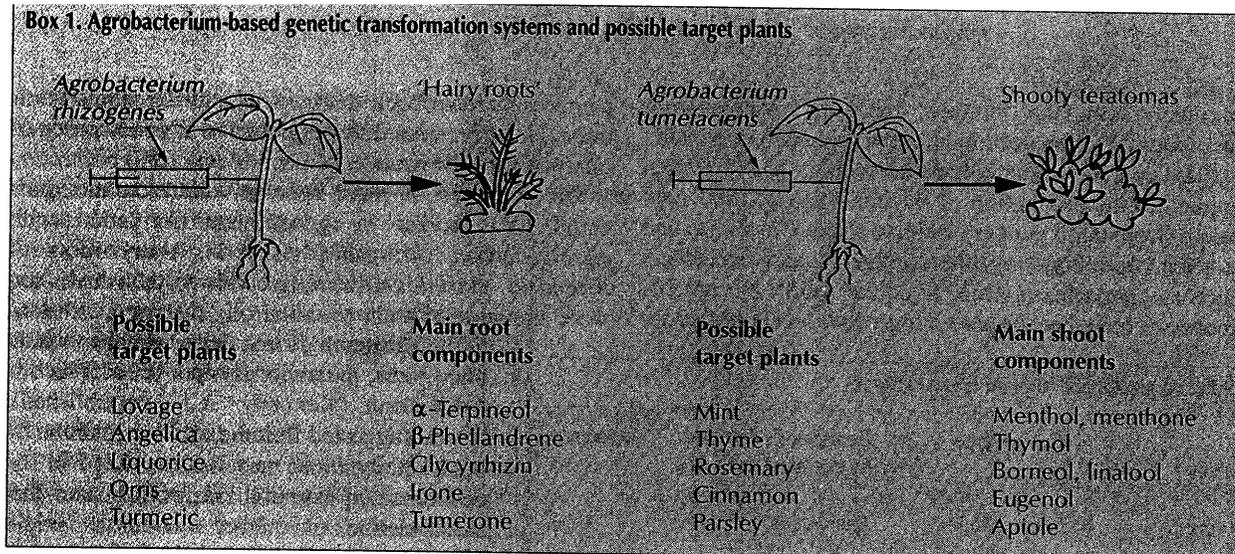
## Alternative plant culture systems

Plant cell and tissue cultures may be established routinely under sterile conditions from, for example, leaves or stems excised from the whole plant. Under the appropriate environmental conditions and in contact with defined solid nutrient media, different types of cultures can be induced to develop: disorganized masses of undifferentiated cells ('callus'), or differentiated shoot, somatic embryo or root cultures. Shoot and root cultures can also be induced by genetic transformation with certain plant pathogenic bacteria; some types of *Agrobacterium tumefaciens* can induce rapidly growing shooty 'teratomas', and *Agrobacterium rhizogenes* causes 'hairy roots' to develop at the site of contact with the plant (Box 1). Upon dispersion in liquid media, callus forms suspension cultures that can be grown to very large volumes in bioreactors similar to those used for bacterial and yeast growth. The more differentiated culture systems require different handling in liquid culture and can, as yet, only be grown in relatively small fermenters (Fig. 1).

## Flavours from plant cell and tissue cultures

An attempt to commercialize a flavour-production process based on plant cell cultures has been made by Escagenetics Corp., CA, USA. The company holds a patent describing a plant cell culture system for the production of vanilla flavouring, the main components of which are simple phenolic compounds including vanillin and vanillic acid<sup>1</sup>. The company has used conventional methods (addition of charcoal or ion-exchange resins to the medium) in an attempt to enhance the production of vanilla flavour, and have obtained yields of nearly 100 mg vanillin per litre of culture. In terms of biomass, this yield would have been ~14 mg vanillin per gram of cells (dry weight). The maximum yield of vanillin was obtained after ~12 days. Although attaining this yield may be a step in the development of a commercial process for the production of vanilla flavouring, it is likely that a several-fold increase in yield would be necessary for production to be economic.

A large proportion of flavour compounds consist of monoterpenoids. Research suggests that the accumulation of monoterpenes is almost always associated with the presence of highly specialized structures containing secretory and accumulatory elements: oil cells, glandular trichomes, oil or resin ducts, or a glandular epidermis. Terpenoid compounds are relatively cytotoxic, and a



compartmentalization mechanism is essential to permit accumulation to reasonable levels; in plants, the physiological function of such components may frequently be associated with defence.

Plant cell cultures, which do not generally have specialized accumulation sites, are not able to accumulate high levels of monoterpenoids. Consider, for example, the case of anise (*Pimpinella anisum*). The fruit is the main natural source of anise oil, the main component of which is *trans*-anethole (70–90% of the total oil content). Callus cultures, containing low amounts of essential oil (only 40 mg per kilogram, fresh weight; ~1000 times less than that found in the fruit) have flavour profiles more similar to those of the roots of the anise plant, which also contain relatively high amounts of  $\beta$ -bisabolene<sup>2</sup>.

The major essential oil products of a range of plant culture systems are listed in Table 1. Using capillary gas chromatography, we have observed a wide variation in the essential oil profiles of callus and suspension cultures. Culture age appears to be an important factor in determining whether or not particular products are present, and the cell line used has a significant effect on the oil profile. Furthermore, the extraction method is of critical importance in determining essential oil profiles. However, the consistent finding is that, in the absence of precursors, undifferentiated cell cultures are usually only capable of accumulating very low, uneconomic levels of essential oils (Cresswell, R., Rudge, K. and Stafford, A., unpublished).

As higher levels of differentiation are imposed upon tissue cultures, the performance of the culture systems frequently improves. Numerous shoot cultures that produce essential oil have been reported. A culture system of *Pelargonium* readily regenerated shoots from callus cultures under the influence of particular hormone combinations<sup>5</sup>. Significantly, shoot morphogenesis could be induced in submerged cultures; a morphogenic callus produced green cell aggregates in shaking flasks containing

a liquid medium. Under conditions of a 16-hour light period and relatively low levels of hormonal supplementation, gland-bearing shoots were induced to proliferate; these cultures remained stable over a period of two years. An interesting culture system for the production of essential oil was described by workers at the Institute of Food Research in Norwich, UK, who induced shooty 'teratomas' of the mint species *Mentha citrata* (bergamot mint) and *Mentha piperita* (black peppermint), and examined the essential oil profiles<sup>3</sup>. The induction method involved a genetic transformation step using *Agrobacterium tumefaciens*. However, although glandular hairs do appear to enhance monoterpene accumulation *in vitro*, and, in certain cases, the profile of essential oils is similar to that found *in vivo*, the yield is still low relative to that found in the whole plant. Also, in submerged culture, a large proportion of the product is

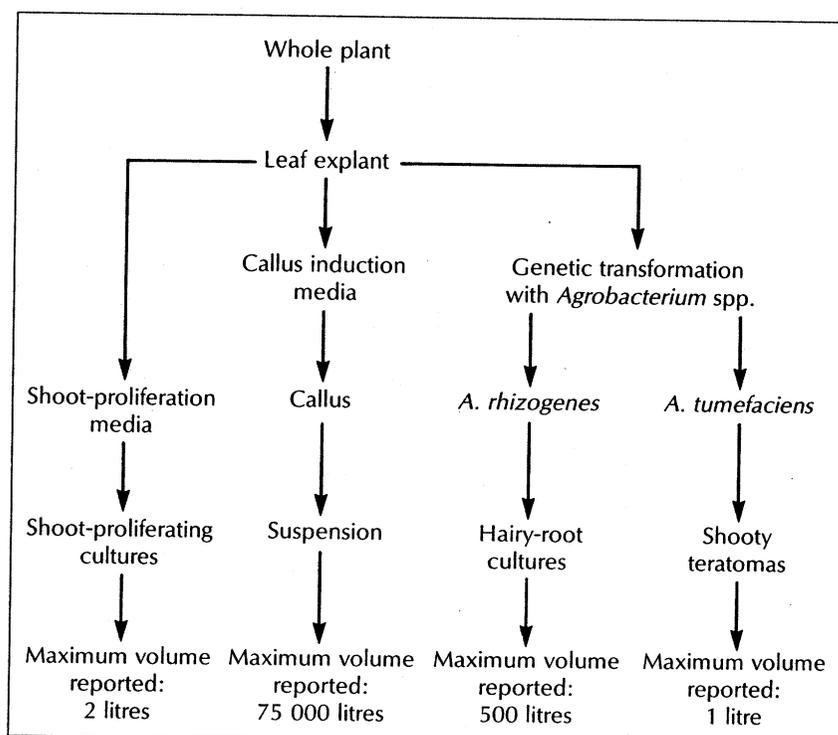


Fig. 1

The various methods of plant culture.

excreted into the culture medium, necessitating incorporation of an accumulating matrix such as a resin.

Plant cell and tissue culture technology has also been applied to the production of flavours such as 'hot' capsaicin (from chilli pepper), quinine (from *Cinchona*) and  $\alpha$ -acids (from hops) as bittering agents, and sweeteners such as glycyrrhizin (from liquorice root)<sup>4</sup>. No notable successes have been reported; only low levels of flavour

compounds have been detected in undifferentiated cultures. However, root cultures could be a good source of many of these flavours.

Root cultures of plant species with aromatic roots could be obtained by genetic transformation with *Agrobacterium rhizogenes* (Box 1). Hairy roots of *Artemisia absinthium* were recently established, and were shown to contain essential oils that were substantially different from those of the whole plant roots, the major component of which is  $\alpha$ -fenchene (Kennedy, A.J., unpublished). It was suggested that the relative immaturity of the hairy roots may have resulted in the difference in essential oil profiles, and that manipulation of culture conditions might effect a more authentic profile.

Exogenous terpenes have been shown to be rapidly metabolized by cell suspensions to form biotransformation/degradation products. Thus, biotechnological processes, based on plant cell cultures, for the synthesis of an acceptable yield of product may only be feasible if monoterpene products are compartmentalized in a nonpolar organic phase or on a resin.

As in the case of microbial cultures, plant cell cultures efficiently metabolize added substrates, and they should be viewed as a potential source of novel enzymes for industrial biotransformation processes. The fates of substrates of pharmaceutical or agrochemical relevance have been investigated extensively, and a wide range of reactions, including double-bond reductions, the reduction of keto groups to hydroxyl groups, demethylation, glycosylation, and esterification with fatty acids have been reported<sup>7</sup>.

In the food industry, biotransformation of the diterpenoid aglycone steviol has attracted some attention, as the glycoside stevioside is a natural sweetening agent ~300 times sweeter than glucose. Plant cell cultures of *Stevia rebaudiana*, the natural source plant of stevioside, and of *Digitalis purpurea* (fox-glove) have been shown to transform steviol into stevioside; novel glycosides were also detected as products of such transformations<sup>8</sup>.

Monoterpenoid biotransformations have been examined in detail (Table 2), and a range of enzyme activities have been detected. However, the level of substrate loading in many of the studies was relatively low; for commercial production, higher substrate concentrations would be desirable. For example, in a study of valencene transformation to nootkatone in *Citrus* cultures, the best yields reported (20–22%) were obtained using 2.7–5.4 mg valencene per litre of culture<sup>9</sup>. Because of the toxicity of certain

Species	Culture type	Compounds detected by chemical analysis	Reference
<i>Foeniculum vulgare</i> (Fennel)	Shoot culture suspension	Anethole (trace)	3
<i>Pimpinella anisum</i> (Anise)	Callus suspension	Anethole, $\beta$ -bisabolene, myristicin	2
<i>Citrus limon</i> (Lemon)	Callus	$\alpha$ -Pinene, limonene, linalool, geranyl acetate	4
<i>Coriandrum sativum</i> (Coriander)	Callus	Geraniol	4
<i>Pelargonium tomentosum</i> (Geranium)	Shoot culture	Limonene, menthone, isomenthone	5
<i>Mentha piperita</i> var. <i>citrata</i> (Bergamot mint)	Transformed shoot culture	Linalool, linalyl acetate	6
<i>Mentha piperita</i> var. <i>vulgaris</i> (Black peppermint)	Transformed shoot culture	Menthol, menthone	6

Species	Substrate	Product	Conversion (%)	Reference
<i>Citrus limon</i>	Valencene	Nootkatone	22	9
<i>Citrus paradisi</i>	Valencene	Nootkatone	80	9
<i>Cannabis sativa</i>	Geraniol	Citral a	35.2	10
	Nerol	Citral b	46.8	
<i>Nicotiana tabacum</i>	Linalool	8-Hydroxylinalool	16.5	11
	Dihydrolinalool	8-Hydroxydihydrolinalool	14.9	
<i>Mentha</i> spp.	Pulegone	(+)-Isomenthone	10	12
<i>Stevia rebaudiana</i>	Steviol	Steviolbioside, Stevioside	NA	8
<i>Lavandula angustifolia</i>	Geraniol	Geraniol	100	13
	Nerol	Nerol	100	
	Citronellal	Citronellol	100	
NA, data not available				

monoterpenes, which is often apparent at concentrations of ~400 mg/l, the upper concentration limit would have to be determined for each substrate and its product. Many conversions are completed in a very short time (2–6 hours), so cell viability should not present a problem in a batch culture system. However, semi-continuous or continuous culture systems may not be compatible with the addition of toxic substrates.

### Manufacture of pigments

Plant cell cultures have been more successfully used for the production of pigments than for the production of flavours. Carotenoids, anthocyanins and betalains can be synthesized and accumulated in culture to quite high levels (Table 3).

The strategies used to obtain some of these yields are interesting. Anthocyanin production in callus cultures of *Ajuga reptans* was increased to ~3.5–4% (dry weight) by a combination of selecting for resistance to the amino-acid analogue *p*-fluorophenylalanine, and manipulation of the culture medium; cultures could be maintained on a medium containing whey as the only carbon source (Callebaut, A., unpublished). When this medium was used, the anthocyanin yield was the same as that found using a more conventional medium containing refined sucrose as the carbon source.

In another investigation, a herbicide was used to enhance the accumulation of lycopene in cell suspension cultures of *Daucus carota*<sup>15</sup>. Chlorophenylthiotriethylamine (CPTA) was used as the selective agent, and yields of 3 mg lycopene per gram of cells (dry weight) were obtained; untreated suspensions could accumulate only 300 µg per gram (dry weight). Tomato cell suspensions could produce 160 µg lycopene per gram (dry weight)<sup>16</sup>, compared with a yield in whole tomato fruit of 60 µg per gram (fresh weight)<sup>20</sup>. However, while CPTA is obviously an effective means of increasing lycopene production, the use of herbicides cannot be advocated in a food context; the inclusion of herbicides such as 2,4-D in standard plant tissue culture media would also be ruled out. Fortunately, cell cultures can usually be 'weaned off' such undesirable media components, and can often be grown on totally hormone-free media.

An attractive feature of plant cell cultures is the speed with which morphological variants can be selected; visual selection of pigments is comparatively easy. For example, in betalain-producing cell lines of *Beta vulgaris*, it is possible to select cell lines that can accumulate different components of the betalain mixture: betaxanthin-producing cell lines are yellow and betacyanin-producing cell lines are violet (Leathers, R.,

Table 3. Pigment production by plant cell cultures

Species	Pigment	Yield	Culture type	Reference
<i>Beta vulgaris</i> (Beet)	Betaxanthins	0.5%, dry weight	Suspension	Leathers, R. and O'Riordain, G., unpublished
		0.1%, dry weight	Suspension	
<i>Bixa orellana</i> (Annatto)	Bixin	134 µg/g, dry weight	Suspension	Boyd, M.G. and Yeoman, M.M., unpublished
<i>Ajuga reptans</i>	Cyanidin-class anthocyanins	3–4%, dry weight	Callus	Callebaut, A., unpublished
		1.0–2.5%, dry weight	Suspension	14
<i>Vitis vinifera</i> (Grape)	Anthocyanins	3–4%, dry weight	Suspension	Pepin, M.F., unpublished
<i>Daucus carota</i> (Carrot)	Lycopene	0.3%, dry weight	Suspension	15
<i>Lycopersicon esculentum</i> (Tomato)	Lycopene	16 µg/g, fresh weight	Suspension	16
<i>Gardenia jasminoides</i>	Carotenoids including crocin	8 times less than <i>in-vivo</i> levels	Callus	Nawa, Y. and Ohtani, T., unpublished
<i>Phytolacca americana</i>	Betacyanin	NA	Suspension	17
<i>Prunus yedoensis</i>	Cyanidin 3-monoglucoside	NA	Suspension	18
<i>Panax ginseng</i>	Cyanidin-class anthocyanins	2–3%, dry weight	Suspension	19
NA, data not available				

unpublished). In a cell line accumulating both types of betalain, betaxanthins accounted for 0.5%, and betacyanins for 0.1% (w/w) of the cell dry weight. The levels of some pigments in plant cell cultures approach 5% (Callebaut, A., unpublished and Pepin, M.F., unpublished), bringing the production of these compounds within the reach of commercial plant cell culture processes.

### Plant culture systems as sources of enzymes

Plants are a traditional source of many enzymes used by the food industry, including β-amylase from barley and wheat, and the proteases papain (from pawpaw) and ficin (from fig); they have been applied mainly in the dairy and beverage industries.

It would be unrealistic to consider plant cell cultures of cereals, pawpaw or fig as potential alternative sources of such enzymes for industry; the whole-plant sources are far too prolific and the enzymes are far too

inexpensive. However, this does not preclude the use for specialist applications of plant enzymes that are, as yet, undiscovered. Plant cell cultures are a convenient source of enzyme material, and have many advantages in this respect compared with the whole plant. Therefore, they can be handled with ease for screening purposes. For example, plant cell cultures of many species are sources of peroxidase enzymes. The current major commercial source of peroxidase, which is widely used in diagnostics, is the horseradish root. Studies of peroxidase enzymes from plant cell cultures have shown that, in some species, virtually all of the enzyme activity is extracellular. Activities of >200 000 units per litre of culture medium have been obtained (Stepan-Sarkissian, G. and Grey, D., unpublished).

However, culture activity is not the only criterion for selection. If an enzyme exhibits promising characteristics

but the yield of plant cell culture is low, gene-cloning technology presents another option: the gene can be cloned into a bacterial or yeast cell under the regulation of a strong promoter, and the enzyme can be expressed to high levels and secreted into the medium in a rapidly growing culture system. In the long term, expression in a bacterial or yeast system may provide a more economic route for the production of many plant enzymes.

### Novel food ingredients

Plant cell cultures cannot always synthesize and accumulate the same secondary metabolites as whole plants – a frequent finding during the past ten years that has disappointed workers investigating a number of potential pharmaceutical products. This suggests that aberrant metabolic routes may be a characteristic of cell cultures, a hypothesis that has been supported in numerous cases.

To date, more than 80 novel compounds have been isolated from ~30 different plant cell cultures; these compounds include 23 alkaloids, 19 terpenoids, 30 quinones and 11 phenylpropanoids<sup>21</sup>. Some examples are given in Table 4.

Plant cell cultures may be viewed as a potential source of novel food ingredients with, for example, desirable flavour properties. In this respect, an additional advantage of plant cell cultures is that, like microbial cultures, they can be rapidly scaled up to provide biomass for extraction and analysis. Onion root cultures have been reported to accumulate not only the known flavours derived from cysteine sulphoxide, but also novel derivatives with unusual side chains (Prince, C.L., unpublished). Antioxidants are a class of compounds of great importance to the food industry, as they prevent lipid peroxidation during food storage. Sesame oil and seeds are known to contain several types of lignan compounds with antioxidative properties; novel glycosides with potent antioxidant activity have recently been found in sesame callus cultures (Mimuri, A., unpublished).

### Industrial-scale production processes

Clearly, some products (e.g. essential oils produced *de novo*) are not likely to be obtained from undifferentiated cultures, despite evidence that the monoterpene and sesquiterpene biosynthetic pathways can function in such cultures. Furthermore, the difficulties encountered in scaling up (so far, only fermenter volumes of up to 2 litres have been reported) of inducing sufficient levels of synthesis, and of devising efficient accumulation systems make submerged shoot-proliferation systems commercially unattractive.

An attempt has been made to overcome the potential limitations of submerged systems for the cultivation of organized cultures by using mist-phase systems instead. This approach has been adopted by Wilson and co-workers at the Institute of Food Research, Norwich, UK, in an attempt to scale up the

**Table 4. Plant cell cultures yielding novel metabolites<sup>a</sup>**

Species	Metabolite	Chemical class
<i>Gardenia jasminoides</i>	Tarenosid	Monoterpene
<i>Morinda citrifolia</i>	5,6-Dehydroxylucidin	Anthraquinone
<i>Ochrosia elliptica</i>	Epchrosine	Indole alkaloid
<i>Picalima nitida</i>	Pericine	Indole alkaloid
<i>Ruta graveolens</i>	Rutacultin	Furanocoumarin
<i>Stephania cepharantha</i>	Armorine	Alkaloid
<i>Stephania cepharantha</i>	Norcepharadione	Alkaloid
<i>Thuja occidentalis</i>	Hinokiol	Diterpene

<sup>a</sup> Adapted from Refs 22 and 23

**Table 5. Large-scale plant cell culture processes<sup>a</sup>**

Species	Bioreactor type	Volume (litres)	Products
<i>Catharanthus roseus</i>	Air-lift	85	Indole alkaloids
<i>Lithospermum erythrorhizon</i>	Stirred tank, rotary drum	1000	Shikonin
<i>Digitalis lanata</i>	Air-lift	300	Biotransformation products of cardiac glycosides
<i>Coleus blumei</i>	Air-lift	200	Rosmarinic acid
<i>Rauwolfia serpentina</i>	Stirred tank	75 000	Indole alkaloids
<i>Nicotiana tabacum</i>	Stirred tank	20 000	Nicotine, tobacco biomass
<i>Panax ginseng</i>	Stirred tank	2000	Saponins

<sup>a</sup> Adapted from Refs 22, 25 and 26

cultivation of 'hairy roots'; they have designed a 500-litre fermenter specifically for the purpose<sup>24</sup>. The roots are 'immobilized' on a series of stainless-steel barbs that act as supports, and the growth medium is fed in droplet form from a series of spray nozzles located immediately below the headplate of the growth vessel. The efficacy of this vessel for root growth has not yet been reported, though the same group has observed good growth and secondary product formation in *Datura stramonium* (thorn apple) root cultures in a 2-litre droplet-phase bioreactor system.

The most successful plant culture processes to date have been based upon cell suspensions, and a number of plant cell suspensions derived from different species have been grown to very large volumes (Fig. 1 and Table 5). Whether or not plant cell suspension cultures can be economically used for the production of secondary metabolites depends upon a number of factors, including the market price of the product, market volume, culture growth rate, biomass yield and product yield.

There are few known plant-derived food ingredients that would cost more than £1000 per kilogram, which is often quoted as the 'break-even' point for a plant cell culture process (although this is dependent on the factors given above). The costs of natural vanilla extract and angelica oil (presently ~\$400 per kilogram and \$800 per kilogram, respectively) may approach this value, and the price of Bulgarian rose oil (~\$11 000 per kilogram) is far in excess of this, largely due to the limited supply.

The growth rates of plant cell suspensions vary enormously depending on the species and culture conditions; doubling times of 15–120 hours (much longer than for yeast cultures) have been reported. Cycle times for production purposes may be 7–14 days, and it may take 20–40 days to achieve large-scale volumes. However, technology has now progressed to such a stage that plant cell suspensions can be grown, consistently and without undue problems of contamination, on an industrial scale. High biomass levels can be obtained; up to ~45 g of dry mass per litre of culture has been reported<sup>25</sup>, although such high levels may cause engineering problems. It is possible that new developments in stirring and aeration techniques will enable the exploitation of plant cell cultures at very high densities in industrial processes.

High yields of secondary products have been produced by cell suspensions of many cell lines (Table 6), some of which have been obtained by the selection of highly pigmented callus cultures, as in the case of shikonin.

Although large-scale processes for enzyme production from plant cell cultures have not, as yet, been reported in the literature, it is likely that they will be developed in the future. The productivity, even of unselected cell lines, for certain enzymes cannot be disputed, and the fact that some plant enzymes can be efficiently secreted into the culture medium is an added bonus. The simplicity of plant tissue culture media compared with standard

**Table 6. Yields of selected secondary products from plant cell cultures**

Species	Product	Chemical class	Yield (g/l)
<i>Coleus blumei</i>	Rosmarinic acid	Phenolic	3.5
<i>Coptis japonica</i>	Berberine	Alkaloid	1.4
<i>Lithospermum erythrorhizon</i>	Shikonin	Naphthaquinone	4.0
<i>Morinda citrifolia</i>	Various	Anthraquinone	2.5

microbial or yeast nutrient broths is a particular advantage; for example, it is rarely necessary to add protein to plant culture media, which simplifies the recovery of secreted proteins.

### The future

Capitalizing on the vast experience accumulated over the past decade, we are now in a position to evaluate the potential of plant cell and tissue culture processes for the production of various classes of metabolites and enzymes. Over the next few years, it is expected that many more commercial processes, based either on large-scale plant cell cultures or on microbial systems that express useful enzymes discovered in plant cell cultures, will begin operation.

For the food industry, the most likely targets at present appear to be pigments or enzymes, the latter as either crude extracts or purified preparations for food processing, or as biotransformation agents for the synthesis/improvement of flavour chemicals.

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## Review

# Enzyme engineering

Richard W. Pickersgill and  
Peter W. Goodenough

Protein engineering enables the elucidation of the predominantly non-covalent interactions that confer stability and activity on folded globular proteins. Knowledge of the geometry and energetics of such interactions enables the design of enzymes and other proteins with enhanced properties for pharmaceutical and industrial uses. The development of novel enzymes for food applications presents great opportunities as well as many challenges. One of the major challenges that confronts protein engineers is understanding the factors that determine how proteins fold. Since the processes by which proteins fold are not yet fully understood, it is difficult to determine whether mutant proteins will fold correctly. However, nuclear magnetic resonance (NMR) spectroscopy and X-ray diffraction studies of wild-type and mutant proteins, combined with advanced computational tools and studies of the detailed kinetics of reactions suggest that a solution to the problem of protein folding may be possible.

An enzyme increases the rate of a reaction, but is not changed at the end of the reaction, even though it may be intimately involved during catalysis. The transition state in a reaction is the state with the highest free energy and, consequently, from a thermodynamic point of view, is the least populated. An enzyme catalyses a reaction

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by stabilizing the transition state, thereby reducing the energy of the transition state relative to that of the reactants. Stabilization of transition states by enzymes has been demonstrated by protein engineering<sup>1,2</sup>; such experiments also reveal the characteristics of the interaction responsible for stabilization. Enzyme characteristics such as activity, specificity and stability are controlled by the relative positions of amino acids in the protein and by the physical and chemical properties of the amino acids (which are modified by the local environment within the protein). Such properties are industrially important, since they vary as a function of pH, ionic strength and temperature. Enzymes for industrial use are often required to have activity at low pH and high temperature (e.g. for starch liquefaction). Such conditions enhance the rate of chemical change and inhibit microbial growth. On the other hand, high thermostability may not always be desirable in industrial processes; the first commercial microbial rennets were not inactivated by pasteurization, and less stable enzymes had to be developed. Therefore, the control of activity by the manipulation of temperature or pH is an important aspect of the industrial use of enzymes.

Enzymes can be used to prevent the spoilage of food due to microbial contamination (e.g. use of lysozyme EC 3.2.1.17) and oxidation (e.g. use of glucose oxidase EC 1.1.3.4). The major enzymes currently used in food processing are isomerases, amylases, proteases, lipases, oxidoreductases, pectinases and antimicrobial enzymes. This range may be substantially extended in the future.

### Protein engineering

The structure of a folded protein may be determined by X-ray crystallography (Fig. 1). The interactions involved in substrate recognition and catalysis can then be observed or at least inferred from the structure of the protein and of its complexes. This enables the determination of the key interactions that control substrate binding; subsequently, such interactions can be modified to enhance or diminish the interaction between molecules. Since the factors that influence the process of protein

folding are complex and poorly understood, the result of a protein engineering experiment is not totally predictable. The rationale of protein engineering is to test predictions that are made on the basis of the structure of the folded protein. The final steps in the protein-engineering process are site-directed mutagenesis of the DNA that encodes the protein, expression of the modified DNA, and testing of the mutant protein produced (Fig. 2). The expected result can then be compared with the actual outcome of the experiment, and the assumptions involved in the original prediction can be re-examined in the light of the results. Thus, the links between sequence and tertiary structure, stability, folding intermediates and the function of proteins can be established.

### Modification of protein stability

The stability of a globular protein may be expressed as the difference between the free energies of the unfolded and folded states of the protein in water. The net marginal stability of folded proteins (20–80 kJ/mol) is the sum of several small individual contributions. The hydrophobic interaction is thought to be the primary stabilizing force of the folded state; it accounts for the change in heat capacity of the protein upon folding, and its contribution may be estimated from the surface area that is 'buried' in the folded state. However, the stability of folded proteins cannot be attributed to a single determinant; for example, the hydrophobic interaction alone does not account for the temperature dependence of protein stability, which is the result of a variety of factors involving hydrophobic interactions, hydrogen bonding, electrostatic interactions and disulphide bridges. Some of these interactions may persist, at least for some of the time, in the unfolded state, but the main stabilizing factor of the unfolded state is thought to be its conformational entropy. Amino acid substitutions that reduce the entropy of the unfolded state of the protein (e.g. substituting proline for alanine) may increase the stability of the folded protein solely by affecting the stability of the unfolded protein<sup>6</sup>.

Protein engineering can determine the roles of specific amino acids in making the folded form of the protein more stable than the unfolded form. In practice, this involves the removal of specific interactions observed in the folded state. The removal of a buried CH<sub>2</sub> group in barnase reduced the stability by ~4.6 kJ/mol, and the effects of multiple mutations were shown to be approximately additive<sup>7</sup>. The challenge is to understand the complex factors involved when an interaction is not simply removed, but replaced by a new interaction. For instance, substitutions designed to improve hydrophobic packing by filling the largest cavities in the protein have not always yielded more stable proteins<sup>8,9</sup>. Also, the introduction of disulphide bridges has often not resulted in more stable proteins<sup>10,11</sup>. The stability of proteins has been increased by up to ~7.0 kJ/mol by changing their charge distribution<sup>12,13</sup>, and by more when a number of mutation strategies were combined<sup>14</sup>. Subtilisin (EC 3.4.21.14) was made more stable by almost 16 kJ/mol by combining mutation strategies, resulting in an increase of 14.3°C in the

denaturation temperature and a 300-fold decrease in the rate of thermal unfolding under a variety of conditions.

### Engineering enzyme specificity and activity

The contributions of specific interactions to enzyme specificity and activity, and the possibilities of modifying enzyme activity have been well studied<sup>15</sup>. The specificity of several enzymes, such as tissue plasminogen activator<sup>16</sup>, have been successfully modified, and much research has focused on the pH-activity profile of enzymes<sup>17,18</sup>. Where the pH-activity profile is determined by an ionizable group on the protein, predictive methods can indicate how to tailor the pH-activity profile<sup>19,20</sup>.

### Enzyme stability and activity: industrial requirements

Thermal and chemical stability are major requirements for the industrial use of enzymes. Chemical stability is important because some amino acids are chemically

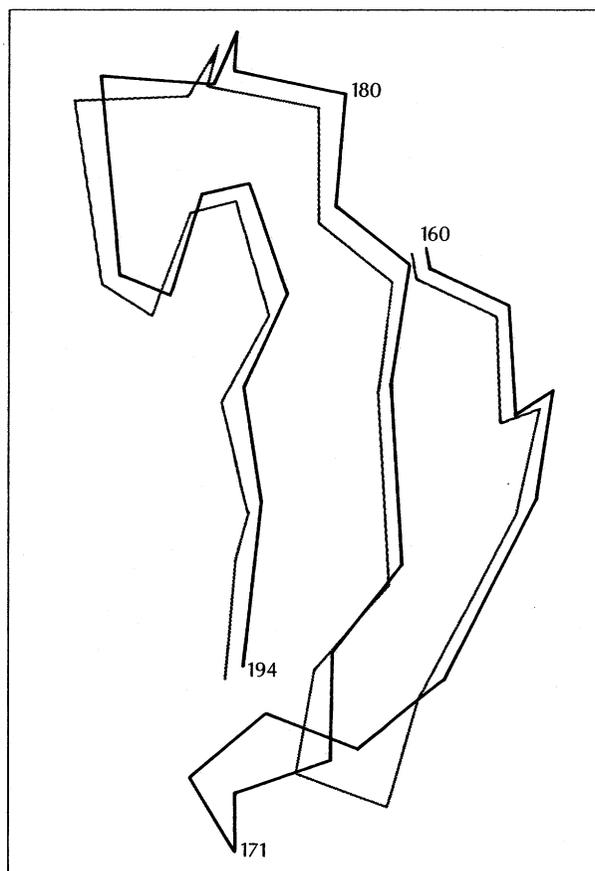


Fig. 1

Part of the anti-parallel  $\beta$  sheet in the C-terminal domain of the cysteine proteases papain (grey) and protease omega (black). This representation of the locations of the  $\alpha$  carbons of the structures illustrates the interactions between non-sequential amino acids that have been established by X-ray crystallography<sup>3-5</sup>, and also illustrates why the native protein is called the 'folded' protein. Note the similarity in the  $\alpha$ -carbon positions of the two structures, except for the loop deletion in papain (for many proteins, loop deletion and insertion is a way of modifying function). Numbers refer to the locations of the  $\alpha$  carbons in the sequence.

reactive or promote hydrolysis of the polypeptide chain. Reactive groups can be removed by protein engineering; a successful example is the removal of a methionine residue in subtilisin to produce an oxidation-resistant subtilisin for use in washing powders<sup>21</sup>. Xylose isomerase (EC 5.3.1.5) catalyses the conversion of xylose to xylulose and of glucose to fructose. The enzyme is the basis for the current industrial production of high-fructose syrups from glucose; immobilized xylose isomerase is an excellent industrial glucose isomerase (Fig. 3). Xylose isomerase can also be used to produce ethanol from xylose. However, the temperature of the industrial process is limited to 60°C, since undesirable side reactions occur at high temperatures and alkaline pH. Consequently, there is a need to modify the enzyme so that its activity is optimal at a pH lower than 6.5, without compromising the thermostability, to facilitate the more rapid reaction rate at the higher temperature. In addition, the conversion of glucose to fructose is more efficient at higher temperatures. The structures of xylose isomerases from several sources have been determined, including that of the enzyme from *Arthrobacter*<sup>22</sup>.

Two magnesium ions involved in catalysis have been identified. The key to maintaining enzyme activity at lower pH may be to maintain the binding of both magnesium ions at lower pH; this hypothesis is currently being tested using protein engineering techniques. Metal binding would be tighter if, for instance, there were more negatively charged ligands to bind the metal ions. Lowering the pKs of existing ligand groups may also be effective. The half-life of xylose isomerase has been increased by replacing chemically reactive lysine residues with arginines (Mrabet, N.T. *et al.*, submitted). Hydrolysis of starch to glucose is best achieved at high temperatures, and more stable amylases may prove

valuable for this process. Amylases that are stable and active at low pH are of industrial interest; the ability to control the number of saccharide units in the product would also be useful, since the properties of the polysaccharide depend critically on its length.

Lipases are very useful industrially because they can be used in non-aqueous solvents to exchange solvent molecules with one of the fatty acid chains of the lipid. In particular, triacyl glycerols are widely used in the food industry; enzymic interesterification of such compounds is well established. Lipases may be used to transesterify inexpensive commercial oils with stearic acid to produce a cocoa-butter substitute. The desirable melting characteristics of cocoa butter are attributable to the positions and proportions of the fatty acids oleate, palmitate and stearate. However, the stability of lipases used in the processing of commercial oils and fats, as opposed to highly purified substrates, is not yet sufficiently high. Recent work on determining the tertiary structures of lipases from *Mucor miehei* and from the human pancreas will enable rational engineering of lipases<sup>23,24</sup>. The lipases studied have a central eight-stranded mixed  $\beta$ -pleated sheet and a highly amphipathic N-terminal helix. The active site is buried under a long loop, and it has been suggested that this loop is opened when the enzyme interacts with lipids. The active site of the lipases is composed of a catalytic triad (Ser-His-Asp) with almost identical geometry to that found in the serine proteases; therefore, the mechanism of hydrolysis is similar to that of the serine proteases. Since lipases act at the oil-water interface, further work is needed to discover how interfacial recognition occurs and to examine the potential for modification of such recognition; protein engineering has been used to investigate interfacial recognition in phospholipase A<sub>2</sub> (EC 3.1.1.4) and to improve the stability of the enzyme<sup>13</sup>. It is likely that the industrial use of

lipid-modifying enzymes will change significantly as a result of the protein engineering of lipases and phospholipases. Serine proteases have been modified to improve their stability in organic (hydrophobic) solvents<sup>25</sup>; since a similar active-site mechanism has been found in lipases, the likelihood of successfully modifying lipases is high.

The replacement of chemical antioxidants by modified antioxidant enzymes is an area of growth in the application of enzymes in the food industry. The oxidation of food constituents such as lipids, flavour molecules, vitamins and colour compounds has become a major problem. In addition to lowering the sensory and nutritional quality of foods, the oxidation of food constituents may lead to the production of 'anti-nutritional' and toxic compounds<sup>26</sup>. Unfortunately, the intake of both polyunsaturated and monounsaturated fats, which need to be protected from peroxidation, is rising, at a time when the use in foods of many effective chemical antioxidants is prohibited.

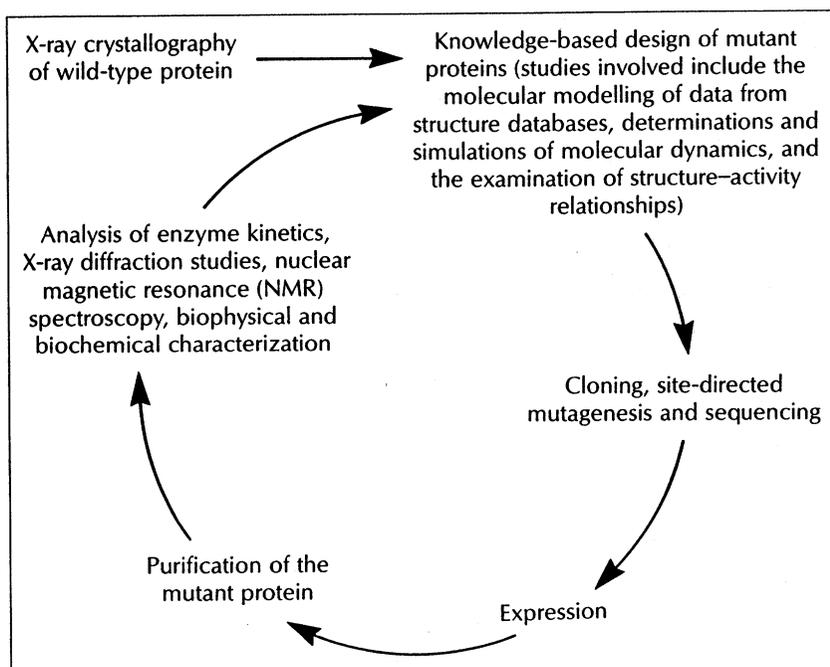


Fig. 2  
Steps in a protein engineering programme.

A number of mechanisms are important in the oxidation of lipids (for a review, see Ref. 27). If all of the pathways are to be inhibited, a mixture of enzymes will be required, including superoxide dismutase (EC 1.15.1.1), oxidases such as glucose oxidase and thiamin oxidase (EC 1.1.3.23), and peroxidases. Such enzymes have different stabilities and environmental restrictions but, clearly, protein engineering offers a way of 'harmonizing' the characteristics of the enzymes so that all may be applied under the same conditions. The tertiary structure and mechanism of action of superoxide dismutase have been known for a number of years<sup>28</sup>, and the structure of the flavoprotein enzyme glucose oxidase is being elucidated<sup>29</sup>. Other flavoproteins, such as thiamin oxidase, have not yet been crystallized. However, intensive research is in progress in Europe to develop enzymes for use as antioxidants in foods; the research programmes include protein engineering programmes in the FRG, the UK and Denmark.

### Difficulties presented by protein folding

As mentioned earlier, a major challenge faced by protein engineers is the uncertainty of whether or not mutant proteins will fold correctly. Folding is the process by which the extended semi-flexible chain of amino acids comes together to form the globular folded protein. The factors that determine how the amino acids in the chain fold into position in the time available and without the chain acquiring strain are poorly understood. Since the time a protein takes to fold is relatively short (of the order of seconds), a chain cannot explore all possible conformations; hence, protein folding is directed to one particular structure.

The results of rapid urea-gradient electrophoresis and hydrogen-exchange experiments suggest that the folded protein becomes unfolded without the formation of partially unfolded states<sup>30</sup>. However, whenever a slow-refolding form of the protein is being studied, the unfolded protein does not remain unfolded at low urea concentrations, but rapidly equilibrates with more compact, though non-native, conformations. Such observations suggest that the rate-limiting step occurs at a very late stage in the folding process and at a very early stage in the unfolding process. The unfolded protein equilibrates rapidly with partially folded conformations when placed under conditions that favour refolding, which suggests that only a small fraction of the many possible conformations are present in the unfolded state. Therefore, protein folding is thought to involve a number of metastable globular intermediates, the 'molten globule' states<sup>31</sup>, that rapidly interconvert.

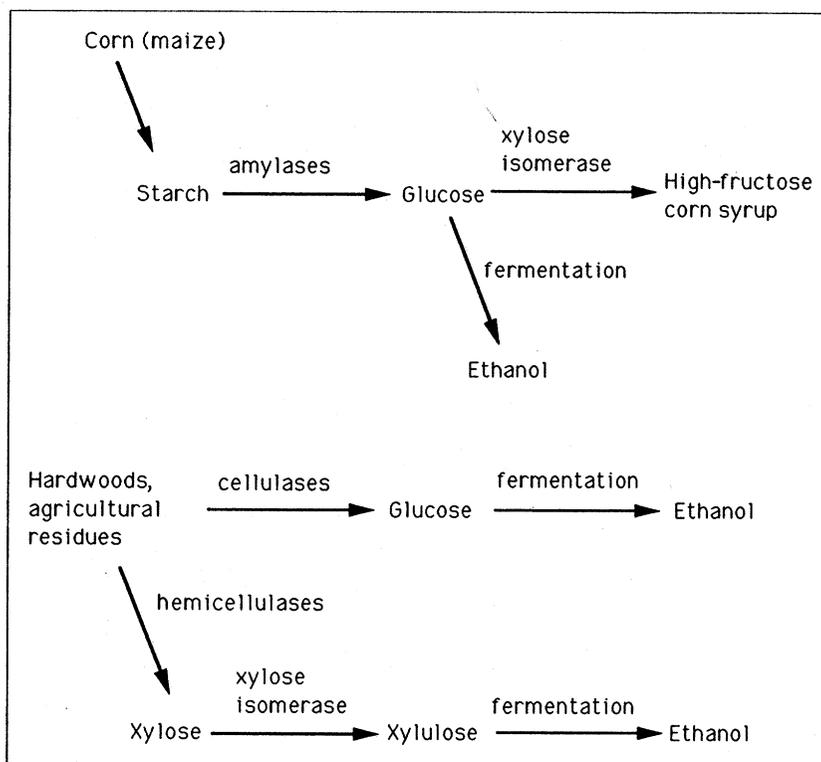


Fig. 3

The industrial uses of xylose isomerase (and some of the uses of amylases, cellulases and hemicellulases).

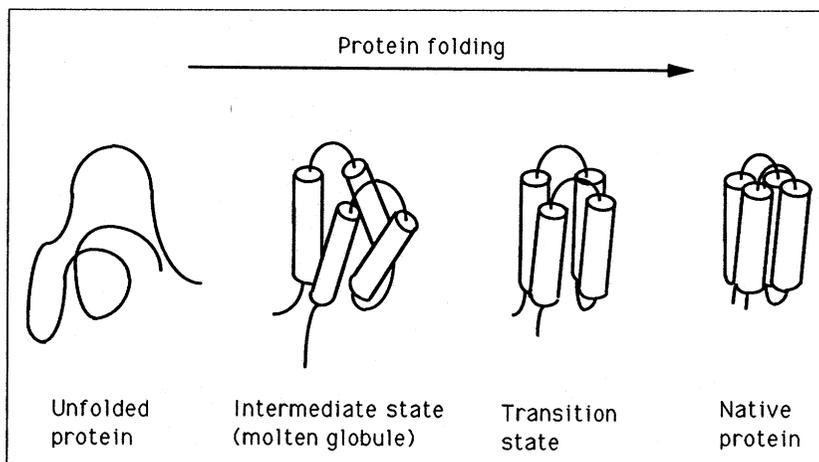


Fig. 4

A schematic representation of the current interpretation of protein folding. Each drawing represents an ensemble of conformations. As the proteins fold, compactness and hydrophobic packing increase, and the number of possible conformations decreases.

Thus, the transition state is a more compact, almost native mixture of conformations. Just as the study of deletion mutants has enabled the evaluation of the roles of specific interactions in determining the stability of the folded state, it is now being used to help to explain the significance of specific interactions to the formation of intermediate and transition states in the folding of barnase<sup>32,33</sup>.

In summary, recent work on protein folding confirms the existence of secondary structure in the intermediate and transition states; there are stronger hydrophobic interactions in the transition state (Fig. 4). The next step

is to stabilize the folding intermediates to enable further evaluation of their structure, and to research the kinetics of the folding process.

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## Cross-disciplinary trends

*Trends in Food Science & Technology* is one of nine Trends journals published in Cambridge, UK. The following selection of recently published Trends articles may be of interest to food scientists and nutritionists.

Pharmacological approaches to appetite suppression,  
by John Blundell,  
*Trends in Pharmacological Sciences* 12, 147–157

Strategies for coping with low environmental temperatures,  
by John Harwood,  
*Trends in Biochemical Sciences* 16, 126–127

A landmark in food-borne parasitic zoonoses,  
by K.D. Murrell, J.H. Cross and C. Harinasuta, *Parasitology Today* 7, 68–69

From sphygmomanometers to synchrotrons: the progress of protein structure determination,  
by Michael J. Geisow,  
*Trends in Biotechnology* 9, 115–116

Biotechnology and intellectual property. Part 1: Patenting in biotechnology,  
by R. Stephen Crespi,  
*Trends in Biotechnology* 9, 117–122

Substantial evidence has accumulated in recent years that suggests new nutritional roles for certain carotenoids in addition to the value of some as a source of vitamin A. Unfortunately, the commonly used calculation of the carotenoid contents of foods as retinol equivalents does not take the non-provitamin A activities of carotenoids into account. Thus, there is a need to express the content of  $\beta$ -carotene and, perhaps, other carotenoids in foods in milligrams, and to establish a new and separate recommended dietary allowance (RDA) for such nutrients.

Many supermarkets are currently displaying colorful messages above their fruits and vegetables – for example, ‘rich in vitamin A’ over the carrots. However, carrots do not contain vitamin A. They do contain some of the 600 known carotenoids, some of which may be converted to vitamin A;  $\beta$ -carotene is one of ~50 carotenoids that can be converted enzymatically to vitamin A by cells in the human intestine if there is a demand by the body for vitamin A. Several other carotenoids, and  $\beta$ -carotene that is not converted are absorbed, circulate in the blood, and are found in cells and tissues.

Evidence has been accumulating over the past 20 years that  $\beta$ -carotene has biochemical characteristics and physiological activities that are not shared with vitamin A. Similarly, vitamin A has numerous functions that are not fulfilled by  $\beta$ -carotene or any carotenoid. Research on the newly recognized properties of  $\beta$ -carotene suggests that the carotenoid has a critical role in the activities of the immune system, especially in those immune responses that are involved in the prevention of cancers, such as the killing of tumor cells.

Since there is accumulating evidence that carotenoids have unique functions in the generation of immune responses and in other roles, it is important that dietary recommendations reflect such findings. Thus, the carotenoid contents of foods should be expressed in milligrams rather than as retinol equivalents; the calculation of retinol equivalents does not take into account any of the additional functions of carotenoids. Moreover, such calculations often lead to unfounded apprehension by consumers about inappropriately high intakes of vitamin A activity. Thus, a new and separate recommended dietary allowance (RDA) for  $\beta$ -carotene and, perhaps, other carotenoids should be established.

### Vitamin A compared with $\beta$ -carotene

It has been consistently reported in more than 30 epidemiological studies that high intakes of carotenoid-containing foods (mainly fruits and vegetables) and high serum  $\beta$ -carotene levels are associated with lower incidences of certain cancers, especially lung cancer<sup>1</sup>. In contrast, high intakes of foods containing vitamin A

# Non-provitamin A activity of carotenoids: immunoenhancement

Adrienne Bendich

*per se*, such as dairy products and organ meats, have not been associated with a lower risk of lung cancer or of many of the other cancers that seem to be prevented by diets rich in carotenoids<sup>2</sup>. The mechanism for the possible activity of carotenoids in this respect may be immunoenhancement, involving the antioxidant and singlet-oxygen quenching properties of carotenoids;  $\beta$ -carotene is a highly effective quencher of singlet oxygen, which is, technically, not a free radical, but an excited state of oxygen that is capable of producing free radicals. Free radicals and their products adversely affect many immune reactions<sup>3</sup>.

Vitamin A is a relatively poor antioxidant compared with  $\beta$ -carotene, and cannot quench singlet oxygen. Nonetheless, vitamin A has been called the ‘anti-infective vitamin’ for more than 30 years due to its proven importance in the generation of immune responses, especially to pathogens. Recent studies have shown that vitamin A and  $\beta$ -carotene enhance different aspects of the immune response<sup>4</sup>. In addition, immuno-enhancement associated with  $\beta$ -carotene is favored by an optimal vitamin A status<sup>5</sup>.

### A historic perspective

In the 1930s, studies on animals and humans showed that dietary carotenoids reduced the number and severity of some infections. The findings were explained by the fact that the carotenoids served as sources of vitamin A, and that vitamin A was the immuno-enhancing, anti-infective agent<sup>6</sup>.

In the 1950s, investigators at the Karolinska Institute in Sweden discovered that tomato juice enhanced non-specific resistance to infection in animal models. Characterization of the lipophilic fraction of tomato juice showed that lycopene, a carotenoid lacking vitamin A activity, was the active agent. The injection of lycopene into mice significantly increased their resistance to bacterial infections. Vitamin A was ineffective, and the all-*cis* isomer of lycopene was approximately 50% as effective as the naturally occurring all-*trans* isomer. However, oral administration of lycopene was not effective. The injection of  $\beta$ -carotene also protected against infection, but was not as potent as lycopene; a number of other non-vitamin A carotenoids were also

protective. Furthermore, administration of lycopene before an initial infection increased resistance to subsequent infections, and administration of lycopene before X-irradiation protected mice from lethal bacterial infections. Thus, the first suspicion that immune function could be enhanced by carotenoids, and that the enhancement was unrelated to vitamin A activity was reported in 1958.

The Karolinska group also examined the effects of the administration of lycopene on the growth of Ehrlich ascites tumor cells in mice. Pretreatment with lycopene increased the tumor-free period and increased survival time, and injection of lycopene after the administration of certain ascites tumor cells retarded the growth of the tumors. The findings of the Karolinska group were recently reviewed<sup>7</sup>.

In the 1970s, studies comparing the activity of  $\beta$ -carotene with that of vitamin A showed that both nutrients prevented stress-induced involution of the thymus gland. Since the thymus gland is essential for the maturation of T lymphocytes, prevention of the loss of thymic tissue is an indication of the maintenance of immunocompetence. Supplementation with vitamin A or  $\beta$ -carotene increased the number of circulating lymphocytes. Both nutrients also enhanced the rejection of foreign skin grafts by mice<sup>8</sup>; rejection of foreign tissues is a classic indicator of cell-mediated immunocompetence. Cell-mediated immune responses are involved in protecting against viral, bacterial and fungal infections, and are the immune responses elicited by vaccinations. The ability to recognize and kill ('reject') foreign cells or tissues and tumor cells is also a function of cellular immune responses.

## Current research findings

### Laboratory studies

Since the 1980s, research on the immunological effects of carotenoids has focused on the determination of specific effects not associated with vitamin A. Structurally, canthaxanthin is very similar to  $\beta$ -carotene but, due to the 4,4'-dioxo configuration, it does not have provitamin A activity in mammals. The chemical structure of canthaxanthin, which contains nine conjugated double bonds, enables it to act as an antioxidant and singlet-oxygen quencher. Comparisons between carotene and canthaxanthin showed that: both carotenoids enhanced the proliferative responses of T and B lymphocytes to mitogens; both increased the tumor-killing activities of cytotoxic T cells and macrophages; and both stimulated the secretion of tumor necrosis factor  $\alpha$  and, at the same time, lowered the number of tumors<sup>5</sup>.

High levels of  $\beta$ -carotene and canthaxanthin have been found in the spleen of rodents; the thymus also contained  $\beta$ -carotene (thymic canthaxanthin levels have not been measured). Results of serum and tissue analyses have shown that canthaxanthin is not converted to vitamin A. Thus, the immunoenhancement by both carotenoids (which have similar antioxidant and singlet-oxygen quenching capacities, and differing provitamin A activities) in these experiments is attributable to an

effect of the carotenoids that is separate from any provitamin A activity<sup>9</sup>.

Dietary supplementation with canthaxanthin and vitamin A significantly overcame the immunosuppressive effects of exposure to ultraviolet (UV) light, which was not alleviated by supplementation with either compound alone, again suggesting the importance of optimal vitamin A status for the elucidation of carotenoid effects<sup>10</sup>.

### Human cells *in vitro*

Exposure, *in vitro*, of human peripheral blood mononuclear cells to carotenoids resulted in the increased expression of surface markers for natural killer cells and for transferrin and interleukin-2 receptors<sup>4</sup>, increased lysis of tumor cells by natural killer cells, secretion of a novel tumoricidal cytokine, and the protection of macrophages and neutrophils from damage by oxidative products of phagocytic cells. Addition of canthaxanthin or  $\beta$ -carotene to culture media protected monocytes from suppression of phagocytic activity induced by UV light, and resulted in increased tumoricidal activity. The effects of provitamin-A carotenoids in cell cultures can only be attributed to carotenoid effects, since the enzyme required for conversion to vitamin A has not been found in cultured lymphocytes<sup>11</sup>.

### Human studies

Short-term, high-dose supplementation with  $\beta$ -carotene (180 mg per day for two weeks) resulted in an increase in the number of circulating T-helper cells<sup>12</sup>. Supplementation of healthy elderly individuals for two months with 45 or 60 mg  $\beta$ -carotene per day increased the concentration of peripheral blood cells bearing markers for natural killer cells and three markers of mononuclear cell activation<sup>13</sup>.

In another human study, it was found that vegetarians had similar serum vitamin levels to a matched non-vegetarian population; however, serum  $\beta$ -carotene levels were two times higher in the vegetarian group. In addition, natural killer cells from the vegetarian group lysed twice as many tumor cells as did cells from the non-vegetarian group<sup>14</sup>.

### Immunosuppression caused by exposure to UV light: protection by $\beta$ -carotene

Singlet oxygen is formed in humans when UV light hits a photosensitive molecule in the skin. The activated photosensitizer can then cause inflammatory responses that result in tissue damage and the formation of immunosuppressive compounds. Carotenoids are present in human skin, and their concentrations are decreased following exposure to UV light<sup>15</sup>. Exposure to UV light also depresses some immune functions<sup>16</sup>, including delayed hypersensitivity responses, which reflect the overall immune status of the individual<sup>17</sup>.

Recently, in a placebo-controlled, double-blind study, supplementation with 30 mg  $\beta$ -carotene per day before, during and after exposure to UV light significantly protected against the suppression of delayed-hypersensitivity immune responses<sup>18</sup>. Responses to the

delayed-hypersensitivity skin test (DTH) have been consistently shown to predict clinical outcomes, especially in the elderly; patients with poor DTH responses have an increased risk of mortality. Responses to the DTH are considered by many clinicians to be the most reliable indicator of overall immunocompetence<sup>19,20</sup>.

Exposure to UV light, either in sunlight or artificial light, has been associated with an increased risk of skin cancer. Individuals who are immunosuppressed also have an increased risk of skin cancer<sup>21</sup>. Since a major function of the immune system is to recognize and destroy tumor cells, any depression of immune responses caused by exposure to UV light may increase the potential for skin cancer to develop. Studies on laboratory animals suggest that pretreatment with  $\beta$ -carotene or with carotenoids lacking provitamin A activity can protect against skin cancer<sup>22</sup>. However, once skin cancers have been found,  $\beta$ -carotene supplementation may not be able to reverse the condition<sup>23</sup>.

### Critical issues

Food composition tables are valuable resources for establishing dietary recommendations. Unfortunately, the existing tables focus on the vitamin A activity of carotenoid-containing foods; the values are expressed in terms of retinol equivalents (REs). Furthermore, the use of REs as the critical index of the nutritional value of dietary carotenoids does not take into consideration the non-vitamin A functions of provitamin A carotenoids, and does not include the nutritive value of carotenoids that lack provitamin A activity. The result is an underestimation of the levels of carotenoids present in many foods. Such underestimation is especially important when the predominant carotenoid has no provitamin A activity (e.g. in tomatoes, which contain a high concentration of lycopene). Recent studies using high-performance liquid chromatography (HPLC) have shown that fresh green leafy vegetables contain a relatively low level of  $\beta$ -carotene compared with the levels of other carotenoids such as lutein<sup>24</sup>. Thus, it is strongly recommended that food composition tables express the concentration of the most commonly ingested carotenoids in milligrams, in addition to retinol equivalents.

Another critical issue concerns the biological activities of carotenoid isomers. The vitamin A 'value' of provitamin A carotenoids depends on the racemic configuration of the carotenoid, because the *cis* isomers are poorly converted to vitamin A; most natural products contain 20–50% of the *cis* form. Cooking, exposure to sunlight or other sources of UV light, and certain physical treatments and chemical reactions increase the ratio of *cis* to *trans* isomers of carotenoids in foods and synthetic products. Although the *cis* isomers have little provitamin A activity, there are reports that both isomers may have anti-tumor activity<sup>25</sup>.

There is also evidence from preliminary work in our laboratory and from a recently published paper<sup>26</sup> that certain tissues have high concentrations of the *cis* isomers of carotenoids. In addition, an isomerase may be present in the intestine (and/or individual tissues)

that converts *trans* to *cis* isomers, or vice versa (the *cis* isomer is more soluble than the *trans* form).

### Conclusions and overall perspective

Carotenoids, with or without provitamin A activity, have been shown to stimulate the immune responses needed to fight infections and to kill tumor cells. Immunoenhancement by carotenoids depends on optimal vitamin A status, but the immunological activities of vitamin A and the carotenoids are often distinct. Therefore, determination of the biological significance of carotenoids must consider factors other than provitamin A activity. Recommended dietary intakes of  $\beta$ -carotene and other carotenoids with provitamin A activity should reflect more than simply their potential conversion to vitamin A.

This viewpoint has concentrated on the immunologically related functions of carotenoids. The determination of dietary requirements should consider the new research that examines the many systemic effects associated with  $\beta$ -carotene supplementation. There is epidemiological evidence that individuals with high carotenoid intakes have a lower risk of developing certain cancers<sup>24</sup> or cataracts<sup>3</sup>. New reports of the beneficial effects of  $\beta$ -carotene supplementation on the regression of precancerous lesions and the prevention of overt cancer are of interest<sup>7</sup>. Dietary recommendations should also consider the provocative finding that long-term, high-dose  $\beta$ -carotene supplementation reduced the incidence of all major coronary events by half in an 'at-risk' population<sup>27</sup>.

Finally, an important prerequisite to developing useful dietary recommendations will be the determination of the most sensitive index of the requirement for  $\beta$ -carotene (and other carotenoids). For example, the immune system may have a higher requirement for  $\beta$ -carotene than the cardiovascular system; yet the lens of the eye, the skin, or a precancerous cell may be more sensitive to low  $\beta$ -carotene intakes. Such quantitative studies, first in animal models, and then in humans, if possible, are necessary for the rational establishment of RDAs for  $\beta$ -carotene and other carotenoids.

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## Software Review

### PSA-System Version 1.61

OP & P Software Development (Oliemans, Punter & Partners), The Netherlands, 1990.  
\$20 000 for an eight-unit system; software price depends on system configuration

**PSA-System** is a package for the automated collection of sensory data. The sensory tests available are paired comparison, descriptive tests and ranking tests. In addition, difference tests and time-intensity tests can be set up separate from the main system and run through it. Standard results files are in a format suitable for analysis by the statistical package SPSS. A companion program, PSA-Convert, is available for manipulating results files and converting them between a variety of formats for further analysis by common statistical packages. PSA-2, a completely new program, is scheduled for release later this year.

PSA-System runs on a network consisting of a central control computer and up to 24 peripheral or satellite units. The central computer can be any IBM AT-compatible machine. At least an 80286 processor and a 32-Mb hard disk are recommended; a colour display is optional. Two variants of the system are available for Atari 1040 ST- or PC-compatible peripheral units. One or two peripheral units can be connected via the standard serial ports of the central computer; up to 24 can be connected via 'Hostess' serial interface boards. An Epson-compatible printer is required.

PSA-System comprises six main menus with numerous sub-menus. Project files are stored in one of 16

work areas, which are sub-directories of the directory holding the software. The subject and product database tables and configuration files are shared by the work areas. The main menus are 'Subjects', 'Products', 'Prepare', 'Run', 'Process' and 'Miscellaneous'. A list of sub-menus is displayed when a main menu item is selected. When a sub-menu is selected, a list of the active function keys is displayed on the screen. A context-sensitive help system is available.

'Prepare' is the most important menu, which allows the operator to set up experiments. It is subdivided into four sub-menus: 'Experiment file', 'Product file', 'Information' and 'Labels'. The first option enables the operator to create an experiment file, which can contain information about a single test or a combination of tests (up to a maximum of 16). The tests available are paired comparison, descriptive tests and ranking tests. In a paired comparison test, there are four design types, a maximum of six questions and a maximum of 16 products. In a profiling test, up to 98 attributes are allowed, divided over 14 screens, with seven attributes per screen. The maximum number of observations is 768 (the number of attributes times the number of products). There are three different scaling options available for each attribute: a 100-point

pseudo-analog line scale with up to three defined anchors, a category scale with up to ten categories, and a magnitude-estimation scale allowing numbers between zero and 999. In a ranking test, the maximum number of samples is 16 and the number of anchors is two. Two-dimensional ranking is possible, and results are recorded, but anchors are not available for the vertical dimension. It is not possible to edit the information contained in an existing experiment file, but the information can be copied to another file, which can then be edited. Once an experiment has been set up, the operator must confirm all of the information in the file.

Unlike the other PSA-System tests (paired comparison, profiling and ranking), which are prepared in the main menu, difference-test experiment files are prepared with the help of an external program. The 'Difference test module' is an application within PSA-System that prepares from the difference-test file an experiment file that can be used by PSA-System. Using this module, 12 different types of test can be prepared, ranging from one-stimulus tests to three-stimulus tests.

The 'Time-intensity module' is another application within the PSA-System, in which the subject can indicate perceived intensity on the screen of the monitor using a mouse. The input files can be prepared using any text editor if the text is kept as DOS text. The input files can then be run through 'Applications' in PSA-System. The screen shows either a vertical gliding scale or a vertical bar scale with up to

ten anchors. The operator can define the location and text of the anchors. Output files consist of copies of the information in the input files and data points for each sample. This information is reported as three numbers: the sequence number of the data point, the value of the scale on the screen at that moment, and information about the mouse (which button is pressed).

PSA-System has been well designed and implemented, and works well under normal conditions. However, problems can arise when the system is forced to adapt to the procedures used in individual laboratories. For example, a nosing procedure for whisky samples that we have adopted in our laboratory requires a number of subjects to nose the same set of samples; the subjects follow each other along a row of samples. This is difficult using PSA-System. A single experimental file is set up for the profiling of whisky using a fixed vocabulary of 25 descriptors. Eight samples are assessed at each session, so eight different product files must be created for one experiment file (containing the vocabulary, scales, etc.). One sample is placed in each of eight tasting booths and one product file (containing one sample) is assigned to each peripheral unit. Once a subject has completed the testing of a sample, the system removes that product file

from the peripheral unit, and it must be reassigned by the panel operator. Thus, the panel coordinator must be at the keyboard of the control computer for the duration of the session. It would be helpful if there were an option that allowed for automatic reassignment of the same product file to a given peripheral unit. A further inconvenience for experienced subjects is that they must use the mouse to confirm the first (introduction) screen, even when no text is displayed.

Further problems have been encountered with free-choice profiling, where each subject uses a different set of attributes. In this case, a separate experiment file is created for each subject but, although the same samples are assessed by all subjects, a different product file must be created in each case; this is very time consuming, especially for testing large consumer panels. We have also encountered memory problems when running PSA-System under MS-DOS version 4.0 for free-choice profiling. The system will not allow the creation of more than one profiling experiment at a time due to lack of memory. Additional free memory must be made available by not loading software that is not absolutely essential (e.g. DOS drivers).

It is not possible to specify the order of assessment when setting up the product files, since PSA-System randomizes the

order when setting up the experiment. When the operator requires the samples to be examined in a particular order, that order must be written out separately.

From the subjects' point of view, using PSA-System for profiling takes longer than our previous method of collecting data on machine-readable cards. For profiling Scotch whiskies, 25 attributes are used, spread over four screens. A score must be recorded for every attribute whether or not it is present in the sample, so a score of zero is allowed. The subjects can view and edit previous screens, but screens are rather slow to update. In general terms, using PSA-System, the subjects nose each sample more frequently, there is less discussion of results (since the subjects have no data to refer to once they have left the tasting booths), and subjects cannot add text comments about the samples if required.

From the viewpoint of the operator, PSA-System is straightforward to operate and offers a great timesaving in the setting up of experiments and the processing of results. Complex profiling experiments can be set up, the data processed and the results made available within a day.

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## Books Received

**Review copies** of the following books have been received. The appearance of a book in the list does not preclude the possibility of it being reviewed in *Trends in Food Science & Technology* in the future.

K.K. Carroll (ed.) *Diet, Nutrition, and Health* The Royal Society of Canada, 1989. £16.10 pbk/£35.62 hbk (xx + 347 pages) ISBN 0 7735 0733 7 (pbk) 0 7735 0741 8 (hbk)

I. Luis Gomez *High Nitrile Polymers for Beverage Container Applications* Technomic Publishing, 1990. Sw. Fr. 196.00 hbk (xiii + 245 pages) ISBN 0 87762 676 6

B.D. Hames and D. Rickwood (eds) *Gel Electrophoresis of Proteins: A Practical Approach* (2nd edn) Oxford University Press, 1990. £25.00 pbk (xviii + 383 pages) ISBN 0 19 963075 5

V.R. Harwalkar and C-Y. Ma (eds) *Thermal Analysis of Foods* Elsevier, 1990. £55.00 hbk (xi + 362 pages) ISBN 1 85166 436 X

International Programme on Chemical Safety *Selected Mycotoxins: Ochratoxins, Trichothecenes, Ergot* (Environmental Health Criteria Series, Vol. 105) World Health Organization, 1990. Sw. Fr. 29.00 pbk (263 pages) ISBN 92 4 157105 5

Chen Junshi, T. Colin Campbell, Li Junyao and Richard Peto *Diet, Life-style, and Mortality in China* Oxford University Press, 1990. £95.00 hbk (xvii + 894 pages) ISBN 0 19 261843 1

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*Mercantila Publishers Guide to Food Transport: Fruit and Vegetables* Mercantila Publishers, 1989. £45.00 hbk (247 pages) ISBN 87 89010 98 1

Marjorie P. Penfield and Ada Marie Campbell *Experimental Food Science* (3rd edn) Academic Press, 1990. £23.50 hbk (xiv + 541 pages) ISBN 0 12 157920 4

J.R. Piggott and A. Paterson (eds) *Distilled Beverage Flavour* Ellis Horwood, 1989. DM195.00 hbk (x + 352 pages) ISBN 3 527 26887 1

Y. Pomeranz (ed.) *Advances in Cereal Science and Technology, Vol. X* American Association of Cereal Chemists, 1990. US\$80.00 hbk (xiii + 557 pages) ISBN 0 913250 66 X

# *Edible Fats and Oils Processing: Basic Principles and Modern Practices*

edited by David R. Erickson, American Oil Chemists' Society, 1990. \$125.00 (442 pages)  
ISBN 0 935315 30 6

**This is the latest** in a continuing series of proceedings of conferences sponsored by the American Oil Chemists' Society. The focus of the volume is on modern methods of processing edible fats and oils. Starting with a section on the supply and demand situation, the book also covers storage and handling, extraction, refining, nutrition and health, finished products, individual fats and oils, quality control, and meal and by-products, ending with a discussion of waste treatment and environmental concerns in oil processing. The last conference covering a similar range of subjects was held in The Hague, The Netherlands in 1982, so an update should be welcome to those interested in edible fats and oils.

The authors contributing to the latest 'World Conference' come mainly from Europe and North America, with roughly equal representation from each area. India and Malaysia are also represented, due to their expertise with rice-bran oil and palm oil.

Compared with the coverage of the 1982 conference, the range of topics

discussed has been broadened somewhat to include animal fats, marine oils and waste-water treatment. These are timely and useful additions to the material on vegetable oils, which still makes up the bulk of the proceedings. The papers mainly stress current practices, without much in the way of historical review; some new experimental results are also presented.

Some material is redundant, as marketing, storage, handling and processing are covered more than once: first in a general sense, and then in sections on individual fats and oils that discuss the same topics, but with a more narrow focus. This is probably of little consequence, because few will read the volume from cover to cover; its use will be as a reference text, both for knowledge about the latest technology and for information on how problems are handled in other parts of the world.

Since the volume will be used mainly for reference, it is unfortunate that the editor did not choose to prepare an index. The main shortcoming of this excellent source of information is the

difficulty of locating material not identified by a chapter heading.

Discussions held during the conference are summarized for the reader, thus preserving an interesting and fruitful part of the conference. The questioners are not identified, which is not surprising given the difficulty of having questioners state their names, but it would have been useful to include the responders' names.

The conference was held in October 1989. The author of the chapter on supply and demand did an excellent job of including what was, then, timely data; however, when reading the proceedings more than a year later, one wonders how the situation has changed in the intervening months. Perhaps the presentation of material that is quickly outdated should be reserved for the conference itself, with trends rather than current events stressed in the published manuscript.

The papers are well written and edited, and there are remarkably few obvious errors in the book. I find the text helpful for answering questions and as background material for teaching a course on oilseed processing, and highly recommend the volume to others.

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# *Dietary Proteins, Cholesterol Metabolism and Atherosclerosis (Monographs on Atherosclerosis, Vol. 16)*

edited by M. Sugano and A.C. Beynen, Karger, 1990. \$129.90 (US only)/£75.70 (UK only)/DM208.00/Sw. Fr. 174.00 (vii + 163 pages) ISBN 3 8055 5193 2

**It has been known** since 1909 that animal proteins such as those in milk, eggs and meat can induce atherosclerosis in rabbits. The effect has been attributed to the increase in serum cholesterol resulting from feeding animal proteins; however, the mechanisms involved are by no means clear. This book brings together 14 papers that review most of the recent work on the subject, and is based on papers presented at a conference held in Tokyo in October 1988 as a satellite to the World Congress of the International Society of Fat Research. Six of the 14 papers are from Japan, with other contributions from France, the FRG, The Netherlands, Canada and the USA.

Most work on the differential cholesterolaemic and atherogenic effects of proteins have used protein preparations isolated from soybean protein as a 'typical plant protein', and casein as a 'typical animal protein'. Although some studies have been carried out in humans, most have been done using animal models, particularly the rabbit and the rat, in which marked effects are seen. The differences between the cholesterolaemic effects of casein and soybean proteins are less marked in the pig, as pointed out in the paper by C.A. Barth *et al.*, while mice exhibit little or no effect, as pointed out by J. Yamashita *et al.* Unfortunately, the evidence that

soybean protein preparations may be effective in lowering serum cholesterol levels in humans is not critically examined, although some mention of the effect is made in several chapters.

Evidence has now accumulated that indicates that the key to the greater hypercholesterolaemic activity of casein with respect to that of soybean protein is a decrease in the excretion of bile acids and neutral steroids in the faeces. As discussed in the paper by A.C. Beynen, the effect may be primarily due to differences at the intestinal level or at the post-absorptive level. The editors of the volume and their colleagues tend to favour the theory that the primary effect is at the intestinal level. Beynen bases this conclusion on studies of the timing of events; the greater 'digestibility' of casein compared with that of soybean protein may explain the differential cholesterolaemic effects observed. The chapter by Sugano *et al.* presents evidence that a fraction of soybean protein resistant to pepsin digestion binds both neutral and acidic steroids in the

intestine; this increases the excretion of steroids in the faeces, and leads to an accumulation of cholesterol in the liver and serum. The effect cannot be attributed to the saponin content of soybean protein, as an amount of saponin equivalent to that found in soybean protein does not have the same effect.

A number of papers propose that post-absorptive events are primarily responsible for the differences between the cholesterolaemic effects of casein and soybean protein. K. Muramatsu and K. Sugiyama present evidence that dietary cysteine and glycine, either within proteins or as free amino acids, are hypocholesterolaemic, while methionine is hypercholesterolaemic. They suggest that the amino acids may be able to stimulate a variety of post-absorptive events, including the synthesis of very-low-density lipoproteins and the synthesis and conjugation of bile acids. S. Saeki and S. Kiriya propose that amino acids present in dietary casein increase the activity of lecithin-cholesterol acyltransferase (EC 2.3.1.43). According to A. Sanchez *et al.*, the effects can be attributed to changes in the insulin:glucagon ratio that result from the plasma amino acid profiles produced by various dietary proteins; studies of women on diets in which most of the protein consumed was soybean protein or casein suggested that the effects of soybean protein could be attributed to its inhibitory effect on glucagon and the resulting activation by insulin of HMG-CoA reductase (hydroxymethylglutaryl-CoA reductase; EC 1.1.1.88).

**In diagnostic bacteriology**, time is not only money, but also disease, pain and, possibly, death. Hence the continuous pressure to develop rapid, simple and sensitive methods for the unequivocal identification of bacteria. The potential of gene probes in this context stems from the huge advances made in microbial molecular genetics over the past decade, coupled to innovations in diagnostic technology. *Gene Probes for Bacteria* sets out to provide a comprehensive review of this new field. It fulfils the aim most successfully.

The book contains a preface, a general introduction, 17 contributed chapters and an index. Most of the chapters have a clinical/veterinary perspective (e.g. sexually transmitted diseases, or enteric and invasive pathogens); single (but good) chapters cover the detection of bacterial pathogens in food, and the

C.A. Barth *et al.* describe a 'unifying hypothesis', based on the results of studies in pigs. Soybean protein may induce an increase in plasma thyroxine levels, which in turn leads to a number of events in the liver: increased HMG-CoA reductase activity, decreased secretion of very-low-density lipoprotein, increased apoprotein B and apoprotein E receptor activities, and increased bile acid synthesis and excretion. These events may be responsible for the reduction of serum cholesterol levels by soybean protein. However, such results do not rule out the possibility that the metabolic effects observed are secondary to primary events in the intestine; no direct cause-and-effect relationship has been demonstrated. The effects attributed to thyroxine have generally only been seen using much higher concentrations of the hormone than those reported by Barth *et al.*

A paper by Y-S. Huang *et al.* suggests that, in addition to being more hypercholesterolaemic than soybean protein, casein increases the  $\Delta$ -6 desaturation of linolenic acid. A related finding is presented by S. Kemura *et al.*, who found that casein-induced increases in blood pressure and platelet aggregation can be reversed by concurrent feeding of eicosapentaenoic acid.

Studies of the effects of proteins other than soybean protein and casein further complicate the situation. For example, A. Yoshida *et al.* show that rice protein, although a plant protein, is hypercholesterolaemic relative to soybean protein, although it does stimulate the faecal output of acidic (but not of

neutral) steroids. C. Sautier *et al.* reported that the effect of fish proteins on blood cholesterol levels depends on the species from which the proteins are derived. Papers by H. Jacques, and X. Zhang and A.C. Beynen review the importance of matching the dietary fatty acid profile and content and the cholesterol content in such studies. Jacques also presents preliminary results of studies in women that suggest that the replacement of beef, pork, eggs and milk in the diet by lean white fish increases serum cholesterol levels. However, more experiments are required to assess the effects of fish proteins on determining serum cholesterol levels in humans.

One problem with this collection of papers is its lack of cohesion. There is quite a lot of repetition, and not enough attention to the studies that have been carried out in humans. However, the book provides a useful overview of the state of knowledge at the time the conference was held. It is clear that no unified hypothesis exists to explain the differences in the cholesterolaemic effects of various dietary proteins. A hypothesis is presented that explains the differences between the effects of casein and soybean protein; it may become the accepted explanation in the near future, but no hypothesis presently available can adequately explain the cholesterolaemic effects of all the proteins that have been studied.

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## *Gene Probes for Bacteria*

edited by Alberto J.L. Macario and Everly Conway de Macario, Academic Press, 1990.  
\$89.00 (xxiii + 515 pages) ISBN 0 12 463000 6

taxonomy of rumen bacteria. The balance of coverage is probably a fair reflection of the balance of applications of probes to date. Clinically oriented chapters on *Salmonella*, *E. coli* and *Campylobacter* will also be of interest to food microbiologists.

There are 43 contributors, including the editors, all active in the area of gene probes. To avoid any lack of continuity between the chapters, all follow a common format of nine sections: 'Introduction', 'Background', 'Results and discussion', 'Conclusions', 'Gene probes versus antisera and monoclonal antibodies', 'Prospects for the future',

'Summary', 'Materials and methods' and 'References'. The general introductory chapter is an exception to this format. It provides a guide to the book as a whole, and partly offsets the inevitable time delay from writer's desk to bookshelf by citing references that appeared after the main chapters were written.

Each chapter has its own table of contents and cites a wealth of references, and the book is well indexed. The clear layout allows the book to be used in different ways. For example, the 'Introduction' and 'Background' sections can be browsed to get an idea of the problems to which probes are

being applied, and the reasons for their use; the 'Conclusions' sections provide an idea of the success rates of probe assays. The 'Results and discussion' sections cover the strategies in fine detail. Placing the 'Methods' sections at the ends of the chapters means that the detailed protocols (which allow the book to be used as a practical guide) do not interrupt the flow of the main text. Although there is some repetition of material from chapter to chapter, this is more than offset by the benefits of the 'multidimensional' approach, as exemplified in the 'Probes versus antibodies' sections.

The intended audience is anyone involved with diagnostic bacteriology; I believe it has broader appeal, to anyone interested in the use of gene probes as a

diagnostic tool. Many of the principles, procedures and 'take-home messages' are applicable irrespective of the target organism or sequence. For example, the pros and cons of the different methods of probe production and of the different assay formats are considered in both strategic and technical contexts.

The high quality of the text extends to the production, which has resulted in clear type, figures, tables and photographs (some in colour). Also, thankfully, the references include the titles of the papers cited.

The cons of the book are few and do not detract from its value. During the production period, the polymerase chain reaction (PCR) has assumed greater prominence than the text reflects; new work has also emerged on the detection

of bacterial plant pathogens. The 'Summary' sections may have been better placed at the beginning of the chapters, and a final concluding chapter might have proved useful.

This book is immensely readable. As a source of information, it is highly accessible, and as up to date as could be expected. It provides a solid introduction to the subject of gene probes for bacteria, and leaves the lasting impression that, although much has been achieved in this field in a relatively short time, much more is about to happen.

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## *Foodborne Listeriosis (Proceedings of a Symposium on September 7, 1988 in Wiesbaden, FRG)*

Technomic Publishing, 1990. \$49.00/Sw. Fr. 108.00 (156 pages) ISBN 0 87762 795 9

**This book** is an English-language version of the symposium held in 1988. It consists of eight chapters by well-known European authorities on *Listeria*; the final section of the book is a group discussion. Both industrial and academic perspectives are included; however, the predominate theme of the book is the prevention and control of foodborne listeriosis.

The book is useful for its general update on the state of knowledge about *Listeria*, and for the group discussions. The discussions address aspects of methodology and identification, particularly the typing of *Listeria* and the epidemiology of listeriosis. Several key issues are examined, including the question of which indicator organisms (e.g. *L. innocua*) suggest the presence of *L. monocytogenes* in a food. The variability of host susceptibility to the pathogen and the possibility that secondary factors are required for the development of listeriosis are also debated. Another issue addressed is the fact that dairy products, rather than meat products, have been involved in most documented outbreaks of listeriosis; a number of reasons for this observation are suggested.

There are several good chapters in this book, such as that by J. Rocourt ('Identification and typing of *Listeria*'),

which is an excellent summary of current thinking on the classification of *Listeria* spp. L.J. Cox's consideration of the hazard analysis and critical control points (HACCP) approach to limiting contamination of the food supply with *Listeria* represents a contemporary approach to the problem that has gained wide acceptance. Other chapters (e.g. 'The epidemiology of listeriosis in England and Wales', by S.M. Hall, and 'Listeria in the dairy industry in France', by J. Gledal) give an enlightening view of how regulatory agencies and authorities reacted to the *Listeria* issue. These two chapters give the impression that the approach was 'reactive' rather than 'proactive'. Perhaps this is not surprising, given the economic pressures on the soft-cheese market in France. Missing from this book is the North American approach, which

would have added a more international perspective.

There are a number of minor concerns about the editing of the proceedings. Some chapters are difficult to understand in places; they appear to have been poorly translated from the original tongue, since the English versions are laborious to read. This is particularly true of the chapter on *Listeria* in the dairy industry in the FRG. Other distractions are that figures in some chapters are listed as tables; some figures are not numbered at all; and some tables are duplicated. This low-profile editing, while useful in retaining the vitality of the proceedings, is a little irksome.

The book will benefit the less specialized reader, since the various chapters provide valuable references. However, the specialist may have already read most of the presented material in the published proceedings of other symposia or in the original papers.

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### **Letters to the Editor**

*Trends in Food Science & Technology* welcomes letters to the Editor concerned with issues raised either by published articles or by recent developments in the food sciences. Letters should usually be supported by reference to published work.

Please address letters to:

Dr John O'Brien, *Trends in Food Science & Technology*,  
Elsevier Trends Journals, 68 Hills Road, Cambridge CB2 1LA, UK

and mark clearly whether they are intended for publication.

## Dairy Food Thickener

**National Starch & Chemical** has developed a high-viscosity thickener for dairy foods. NATIONAL 78-0148 is an easy-to-cook modified tapioca starch with an especially bland taste, particularly appropriate for delicate-tasting foods.

Easier to cook than many other speciality starches, it develops viscosity very quickly at neutral pH; viscosity development takes slightly longer in low-pH foods. Foods containing NATIONAL 78-0148 that are cooked at neutral pH are heavy bodied and develop a very smooth, short texture.

Its compatibility with milk protein yields rich, creamy products with good stability. Hence, it can be used in both pasteurized and short shelf-life continuously processed dairy products, such as cream and cheese sauces, puddings, cream fillings, and desserts.

For further information, **enter no. 300** on the reader advertising enquiry card.

## New Products for the Separation of Nitrogen from Compressed Air

**New products** that may be used for the production of nitrogen-rich atmospheres for modified-atmosphere packaging have been launched. The products, manufactured by Balston Ltd in the UK and by Air Products and Chemicals, Inc. in the USA, are both based on the separation of nitrogen from air using a membrane filter.

For further information, **enter no. 302** on the reader advertising enquiry card.

## Workshop Launches Microscopy Technique for Food Research

**Research on the structural properties** of foods can now be conducted using a technique known as cryo-field emission electron microscopy (cryo-FEM). The technique was recently introduced to the UK at a workshop run by Hitachi Scientific Instruments and Oxford Instruments.

The advantage of cryo-FEM is that it allows the use of very high magnification without destroying or altering a sample's features. Samples can be frozen in their

natural state and fractured to allow observation of the internal structure. Since low acceleration voltages are used, penetration of electrons into the samples is minimized, which yields superior surface information without damaging the samples. In addition, there is no need to coat the samples, so the image is further improved.

For further information, **enter no. 304** on the reader advertising enquiry card.

## New Photoelectric Sensor

**A new photoelectric sensor** has been launched on the UK market by IMO Precision Control Ltd. The device will be of interest to companies that need to detect objects of assorted colours on the same production line (e.g. the detection of confectionery, such as fruit pastilles or coated chocolate beans, or the detection of pizzas with various toppings).

The new detector, the IMO Omron E3S, uses accurate measurement of the distance of objects from the sensor to detect articles of any colour, against any background. Even

mirror-finish conveyors do not give spurious signals, and the detector does not need different settings to detect different colours – even black. A further benefit is that even small deviations in the position of the object will be detected. The IMO Omron E3S sensor is currently available in two versions: the E3S-LS5 for detection ranges of 4–6 cm and the E3S-LS20 for detection ranges of 4–20 cm.

For further information, **enter no. 303** on the reader advertising enquiry card.

## Plastic Chicken

**Elm Ltd** of Glasgow, UK has developed a product called the 'Plastic Chicken', which offers retailers a low-cost method of monitoring food temperature. The Plastic Chicken, which costs ~£50, is an intelligent temperature-monitoring device with the thermal characteristics of a fresh or frozen chicken. The battery-operated device can be placed in any position in a refrigerated cabinet, and is easy to operate. It can store

temperature readings for three months or more, and stored temperature readings can be passed by infrared signals to a hand-held receiver that displays data on an 80-character liquid crystal display. The receiver can also print tables or graphs of the temperature variations over time.

For further information, **enter no. 301** on the reader advertising enquiry card.

### In next month's issue

Dietary guidelines: their implications for the food industry, by M.C. Nesheim

The effect of dietary garlic on the development of cardiovascular disease, by David Kritchevsky

Rheological measurement of fluid elasticity during extrusion cooking,  
by Mahesh Padmanabhan and Mrinal Bhattacharya

Optimization of deep-fat frying operations, by Michael M. Blumenthal and Richard F. Stier

Role of lipid peroxidation in the mechanism of membrane-associated disorders in edible plant tissue,  
by R.L. Shewfelt and M.C. Erickson