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PREFACE

Proteins perform a number of functions in food. They influence its structure, rheology, and texture. Their dynamic conformational effects on hydration and energy input (mechanical and thermal) contribute an important array of functional properties and form the basis of food fabrication. As sources of essential amino acids, proteins are important nutritional components. The storage proteins of wheat, gliadin and glutelin, which upon hydration form a viscoelastic matrix called gluten, epitomize plant seed-derived functional proteins. This property allows the entrapment and retention of gas during dough proofing and is the basis of leavened bread.

The chapter by MacRitchie describes both the complex physicochemical properties of wheat proteins and the recent research identifying important high molecular weight glutenin components. The loci of gene coding for these proteins are being identified by classical crossbreeding techniques. The advance of knowledge in this area will facilitate the development (via recombinant DNA techniques) of wheat cultivars with the most desirable combination of gliadin and glutenin genes. To this end, methods for isolating specific genes need to be applied, and their insertion and expression in new cultivars, along with the generation of transgenic wheat varieties, need to be perfected. Although genetic engineering techniques have been used successfully with cereals, plant regeneration still remains a challenge with wheat.

Greater success has been found with protein engineering in cotyledonous plants (e.g., legumes such as soybean). This is a key advance because, with the elucidation of the structural and conformational basis of specific desirable physical properties, rational improvements in the functional properties of proteins are now feasible. The review by Utsumi summarizes recent advances in the genetic modifications of plant proteins, including cereals and legumes. This chapter is an excellent introduction to contemporary techniques and strategies for gene manipulation of plant proteins, listing current obstacles to the rapid exploitation of this approach (e.g., knowledge of regulatory genes;

obtaining a high level of expression, gene stability, and plant regeneration).

In keeping with the scope of *Advances in Food and Nutrition Research,* chapters discussing nutrient content, bioavailability, and the metabolic and physiological effects of dietary minerals and fatty acids are provided by Flynn and by McNamara. The value of bovine milk as a source of macrominerals and trace elements and its use in infant formulas are described, thus more precisely defining the safety and adequacy of minerals in formulas, their optimal levels, and how these are affected by other components.

The specific roles of different dietary fatty acids in the etiology and progress of arteriosclerosis and coronary artery disease have not been adequately considered because of the oversimplified approach of categorizing dietary fat as either saturated or unsaturated. The chapter by McNamara presents a comprehensive overview of the relevant literature concerning the effects of various dietary fatty acids in plasma lipoproteins, discussing both the danger of extrapolation from dietary studies and the disparity between animals and humans. Prolonged adherence to diets designed to reduce the average consumer's plasma cholesterol is of marginal significance in terms of increased longevity; however, it may markedly increase the quality of life and also reduce chronic age-related illnesses. New information on the beneficial roles of different unsaturated fatty acids suggests the potential for the food industry to produce modified food products that effectively contribute to health promotion. This is a topic that will receive increased emphasis in the future.

JOHN E. KINSELLA

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PHYSICOCHEMICAL PROPERTIES OF WHEAT PROTEINS IN RELATION TO FUNCTIONALITY

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I. INTRODUCTION

Protein comprises some 8–16% of the grain in wheats grown under normal conditions. In processing the grain or its milled products, this protein generally plays a dominant role, for example, in determining the properties of doughs for breadmaking. Because of the wide variability in functional properties among different flours, considerable research has been aimed at understanding the relations between these properties and the protein composition.

This has not been an easy task, first because wheat protein is a very complex mixture of proteins. Second, it has not been possible to solubilize the total protein in order to characterize it without altering it chemically. Nevertheless, progress has been made in relating protein composition to functionality. Several approaches have been used. The one most widely used has been to survey large numbers of wheat varieties and to test the correlations of functional parameters (e.g., dough extensibility) with different measures of protein composition (e.g., the presence of specific electrophoretic bands).

Another approach has been to separate protein fractions and directly test their contributions either by addition to a control flour or by interchange of equivalent fractions between flours. A recent innovation has been the use of genetic variants in which particular groups of proteins vary in amount, allowing an assessment of their contributions. A further indirect yet useful approach is to utilize information from other areas of science, e.g., studies of the physical properties of polymers and the way these depend on molecular constitution.

One result that has been well established is that the properties of flours, such as their dough rheology behavior, are largely determined by the wheat genotype. This simply reflects the fact that these properties, in turn, are determined by the proteins, whose synthesis is under genetic control. Environmental conditions during growth of the plant and sometimes during the postharvest period can also affect the flour properties, but this influence is normally secondary to that of the genotype.

The practical objective in regard to food science is to breed and grow wheat varieties in which the protein composition is optimum for the properties required in the particular end-use product, including nutritional properties. Attainment of this objective involves a number of disciplines, including both physical and biological sciences. The first step is to define the protein composition for the end use being considered. This has been one of the central tasks to be tackled in cereal chemistry for many decades. It requires the application of biochemistry and the physical sciences to study the proteins and to understand the relations between composition and material properties. In recent times, the chromosomal location of the genes controlling the synthesis of the major groups of wheat proteins has been elucidated. This, together with the knowledge gained from protein composition/functionality studies, makes it possible to devise breeding strategies for modifying flour properties in desirable ways, using genetic manipulation based on careful selection of parents and testing of progeny. Of course, obtaining desirable end-use properties is only one of the considerations for the plant breeder, who also has to plan for yield, disease resistance, and other agronomically desirable qualities.

Although the primary focus of this article is the physicochemical properties of wheat proteins, the aim is to discuss the subject with the integrated picture in mind. Figure 1 is a simple illustration of this integration. Physicochemical

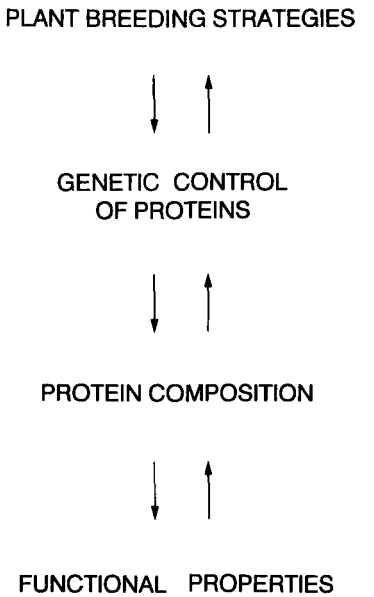


FIG. 1. Integrated picture of wheat quality improvement showing the different steps between relating functionality to protein composition and devising breeding strategies.

approaches are relevant to the lower two of the four levels, i.e., to determine protein composition and to explain the relations between composition and functionality. This knowledge then opens the way for devising wheat breeding strategies to introduce the correct balance of genes for the protein composition required to give the desired grain or flour properties.

II. SOLUBILITY PROPERTIES

One of the major obstacles to the characterization of wheat proteins has been the lack of methods to solubilize the total protein. Measurement of important properties such as molecular weight and molecular weight distribution requires the protein to be in solution, and this has not usually been possible, at least for the most insoluble portion of the protein. Insolubility has also hindered accurate quantification of the main groups of wheat protein using quantitative separation techniques.

A. GENERAL SOLUBILITY PROPERTIES

On the basis of chemical composition, there are two broad classes of proteins in wheat—the albumin/globulins and the gliadin/glutenin, or gluten, proteins. The distinction between albumins and globulins is not always clear. According to the traditional definition, albumins are soluble in water and globulins are soluble in dilute salt solution. This definition does not have a very fundamental basis, as variation of temperature can alter the class to which a given protein is assigned. Whereas albumin/globulin proteins are readily soluble in aqueous solution (except for some of their polymeric forms, considered in Section III), the gluten proteins present problems for their solubilization. Two main factors appear to be responsible. The first is the paucity of ionizable amino acid side chains. Like other proteins, gluten proteins have minimum solubility at a pH near their isoelectric points. As the pH is made more acid or alkaline (thus increasing the net positive or negative charge), solubility increases up to a limiting value. The behavior is illustrated in Fig. 2. However, complete solubilization is usually not attained and some 10–30% of the protein remains undissolved, depending on the particular gluten sample. Repeated extractions can reduce this further, but a proportion of residual protein always remains (MacRitchie, 1987). Two results are notable. One is that the insoluble protein is the highest molecular weight polymeric protein, principally glutenin. The term polymeric is used here to indicate that these proteins are polymers formed from a number of different polypeptide chains by intermolecular disulfide bond linkages. This distinguishes these proteins from the other main group of gluten proteins, the gliadins, as well as from most albumin/globulins, which consist of single polypeptide chains and

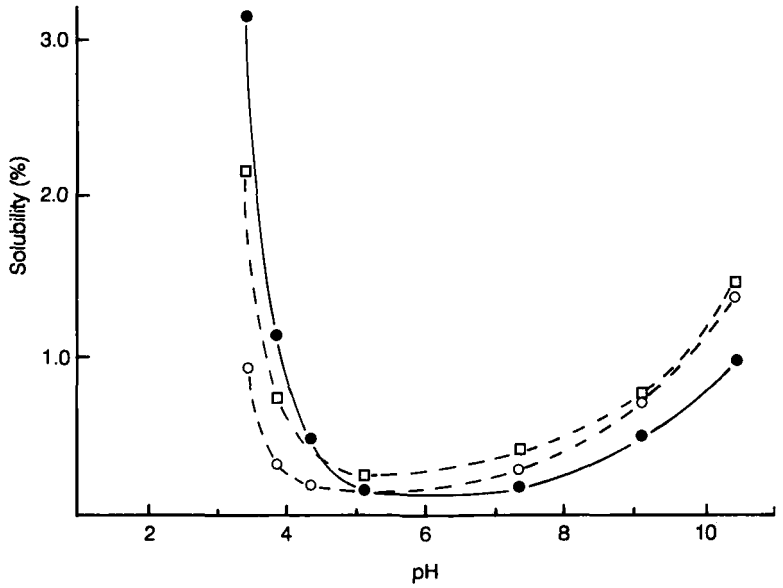


FIG. 2. Solubility and pH relations for gluten protein from different flour samples (MacRitchie, 1979). For these measurements, gluten containing 2.0 g of protein was homogenized with 60 ml of buffer solution for 2 min and the protein concentration was determined by absorbance of the supernatant at 278 nm. Different symbols represent flours of three different varieties.

are thus termed monomeric. The stronger the dough properties of the parent flour, the more protein remains in the insoluble residue. This has been the basis of the residue protein test (Orth and Bushuk, 1972), an early-generation test for assessing dough strength in breeding lines.

Another way of solubilizing gluten proteins by increasing the net electrical charge on the molecules is by using solutions of synthetic ionic detergents. Thus, solutions of positively charged cetyltrimethylammonium bromide (CTAB) and negatively charged sodium dodecyl sulfate (SDS) are among the best solvents for gluten proteins. These detergents evidently associate with protein molecules by hydrophobic and electrostatic interactions. The resultant effect is to confer positive (CTAB) or negative (SDS) charges to the complexes. Although solubility is increased appreciably, a significant proportion of the protein is still not solubilized by these solutions. Similar results are obtained with concentrated urea solutions, which have been frequently used to solubilize gluten proteins (Pomeranz, 1965). A combination of urea (3 *M*), CTAB (0.1%), and acetic acid (0.1 *M*), referred to as AUC (Meredith and Wren, 1966), has been a popular solvent giving high (e.g., 80–90%) but not complete solubilization.

B. FUNDAMENTAL ASPECTS OF SOLUBILITY

In contrast to the intensive focus on solubilization for characterizing the gluten proteins, there has been little systematic basic study of their solubility properties. Mostly there has been an ad hoc approach to choice of solvent. One of the difficulties is that the gluten proteins are a heterogeneous mixture of proteins with different molecular and physical properties, and fundamental solubility studies ideally require pure compounds.

Basically, a substance dissolves in a solvent if this is accompanied by a decrease of free energy (ΔG) of the system (solute + solvent). Changes of free energy are made up of two main contributions, viz.,

$$\Delta G = \Delta H - T\Delta S \tag{1}$$

where ΔH is the enthalpy (or heat content) change, ΔS is the entropy change, and T is the absolute temperature. Molar quantities are normally used in Eq. (1). In simple terms, ΔH is the energy change when solute and solvent molecules interact and ΔS measures the accompanying changes in randomness or disorder. In any solution process, there is an increase in entropy (and therefore a decrease in free energy) as a result of the mixing of solute and solvent molecules. This entropy of mixing is a significant driving force for dissolution. However, if the solute consists of big molecules, such as the largest glutenins, this term becomes relatively small because much less mixing (and less increase of randomness) is achieved than for the same amount of solute made up of smaller molecules. If there are other contributions to the free energy, such as a positive ΔH or other entropy changes that are negative, the entropy gained on mixing will not be large enough to compensate and molecules will not dissolve. This is the reason why the highest molecular weight glutenin is so difficult to solubilize.

Because proteins are copolymers, the enthalpy of solution is a complex function because each of the different amino acid residues interacts with solvent molecules in different ways. The final value of ΔH not only depends on the types of residues present but also the molecular configuration of the molecule, which in turn is determined by the sequence of residues and steric restrictions on chain folding. A simple yet general rule for solubility is that like dissolves like. This usually means that if molecules of solute and solvent are similar, the ΔH term is very small and solubility is facilitated by the entropy of mixing. This may explain the effectiveness of urea solutions. The urea molecule is similar in structure to the polypeptide backbone of proteins. The polar amino acid residues of gluten proteins tend to confer solubility in aqueous solution. In particular, the ionizable amino acid side chains in their charged form interact strongly with water molecules, with a resulting decrease in free energy. However, as mentioned above, the frequency of ionizable amino acid residues in gluten

TABLE I

SOLUBILITY-RELATED PARAMETERS FOR PROTEINS[a]

Protein	NPS	$H\Phi$ (kJ/residue)	p	CHF	RSH ($\times 10^3$ Å$^2/M_r$)	Ref[b]
Glutenin	0.34	3.87	1.16	0.13	—	1
Gliadin	0.39	4.19	1.07	0.08	—	1
Myoglobin	0.32	4.56	1.12	0.34	23.0	2,3
Hemoglobin	0.35	4.51	0.87	0.27	33.8	2,3
Ovalbumin	0.34	4.64	0.92	0.24	41.8	2,3

[a]Abbreviations: NPS, nonpolar side chain frequency; $H\Phi$ average hydrophobicity; CHF, charged group frequency; p, polarity ratio V_e/V_i (external shell and internal volumes, respectively); RSH, relative surface hydrophobicity.

[b]References: 1, Ewart (1967); 2, from Melander and Horvath (1977); 3, from Bigelow (1967).

proteins is low (i.e., some 10% of the total residues) compared to other proteins (see Table I).

C. THE HYDROPHOBIC EFFECT

The other main group of amino acid residues in proteins, the nonpolar side chains, accounts for 30–40% of the amino acid residues. It is the presence of these side chains that hinders solubilization in aqueous solution, the free energy of interaction with water molecules being positive. Contrary to what may be expected, the positive free energy is due to a negative entropy of interaction and not a positive enthalpy. The lack of solubility of nonpolar groups in water is known as the hydrophobic effect. Water is a highly structured material because of the hydrogen bonds that link individual molecules to each other. The introduction of a solute disrupts this structure, as hydrogen bonds have to be broken. If the solute is polar, new hydrogen bonds may form with the solute, but if it is nonpolar, they are unable to be formed. It is postulated that water molecules at the surface of the cavity created by the nonpolar solute rearrange in order to regenerate the broken hydrogen bonds. The consequence is a higher degree of local order than exists in the pure water and therefore a decrease of entropy (Frank and Evans, 1945; Nemethy and Scheraga, 1962).

D. STRUCTURAL PARAMETERS RELATED TO SOLUBILITY

Several useful parameters have been proposed to try to explain solubility properties of proteins in terms of their composition (Table I).

1. *Frequency of Charged Groups*

The frequency of charged groups (CHF) refers to the proportion of amino acid residues in a protein that are charged at about pH 6 (Bigelow, 1967). It is simply the sum of aspartic and glutamic acids, histidine, lysine, and arginine, and the result is expressed as a fraction of the total number of residues. Variation in this parameter is shown for some proteins in Table I.

2. *Nonpolar Side Chain Frequency*

Another simple parameter is the frequency of nonpolar side chains (NPS). This is defined as the number of nonpolar residues of the molecule divided by the total number of residues. The nonpolar residues comprise tryptophan, isoleucine, tyrosine, phenylalanine, proline, leucine, and valine. NPS values of 0.39 for gliadin and 0.34 for glutenin have been calculated (Bushuk and MacRitchie, 1989). These values are relatively high but not sufficiently so to account for the low solubility compared to other proteins. Many soluble proteins have similar NPS values (Bigelow, 1967), as seen in Table I. Waugh (1954) found that a large number of proteins had NPS frequencies between 0.21 and 0.47.

3. *The Polarity Ratio*

Simple consideration of NPS frequency may not be meaningful, as protein molecules tend to fold up in aqueous solution in order to minimize interaction between nonpolar residues and water molecules. This results in a molecular configuration in which the outer shell of the molecule is formed mainly by polar side chains, with the nonpolar side chains concentrated in the center. Based on this model, Fisher (1964) defined a polarity ratio $p = V_e/V_i$, where V_e and V_i are the external (shell) and internal volumes, respectively. Values of p for different proteins were then evaluated by using specific volumes for amino acid residues and assuming that all polar residues were in V_e and all nonpolar ones were in V_i. For this purpose, it was assumed that arginine, histidine, lysine, aspartic, and glutamic acids (and their amides) and tyrosine, serine, and threonine have polar side chains and all other amino acids have nonpolar side chains. Values of p for some proteins are given in Table I.

4. *Average Hydrophobicity*

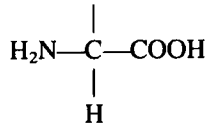
Although the concept of the polarity ratio is valuable, the definition of amino acid side chains as either polar or nonpolar is too simplistic. Obviously there are degrees; e.g., leucine (with four carbon atoms in the side chain) is more

nonpolar than valine (with three carbon atoms). In order to correct this fault, Bigelow (1967) evaluated hydrophobicities of amino acid side chains based on a method introduced by Tanford (1962).

If the solubility of phenylalanine, for example, is N_{HOH} in water and N_{EtOH} in ethanol, then the free energy of transferring 1 mol of phenylalanine from an aqueous solution (at a given concentration) to an ethanolic solution (at the same concentration), assuming ideality, is given by

$$\Delta G_t = -RT \ln N_{EtOH}/N_{HOH} \quad (2)$$

ΔG_t, the transfer free energy, is an approximately additive function of molecular structure. ΔG_t for phenylalanine can thus be considered as the sum of two terms, one for the benzyl side chain and one for the backbone, i.e.,



The transfer free energy for the backbone should be similar to that for glycine, which can also be evaluated by Eq. (2) using corresponding glycine solubility data. Subtraction then gives the transfer free energy for the side chain itself. From experiment, ΔG_t (phenylalanine) is found to be -8.3 kJ and ΔG_t (glycine) is -19.4 kJ. Therefore, the free energy change when benzyl groups are transferred from ethanol to water should be $-8.3 - (-19.4) = 11.1$ kJ. Using this approach, transfer free energies have been derived for all amino acid residues (Damodaran and Song, 1987; Iqbal and Verrall, 1988). These quantities are usually referred to as hydrophobicities (Dunnill, 1965). The average hydrophobicity ($H\Phi$) of a protein is then the sum of the hydrophobicities of all its constituent amino acid residues divided by the total number of residues.

Comparison of average hydrophobicities of proteins (cf. Table I) has not proved particularly successful for explaining solubilities of proteins. This is not altogether surprising, as additional factors are involved. Leaving aside the interactions between solute and solvent, the configuration of maximum entropy (and therefore minimum free energy) for a polymeric molecule is a random coil. Most proteins, however, tend to fold up in aqueous solution in order to minimize contact between their nonpolar residues and water molecules. A compromise is thus reached between the competing tendencies for minimum configurational free energy and minimum free energy of hydrophobic interaction. The nature of the folding process and the reduction of free energy achieved by it depend not

only on the average hydrophobicity, but on the sequence of amino acid residues and steric constraints on chain folding. The size of the molecule is also relevant, as this relates to the ratio of surface area to volume and governs the number of hydrophobic residues that can be buried in the interior of the molecule. Although the model for the polarity ratio has approximate validity (i.e., polar residues in the surface shell and nonpolar residues in the interior), it is too idealistic and it has been well established that dissolved proteins tend to have many of the nonpolar residues exposed or partially exposed to water. It follows that properties such as solubility and chromatographic behavior of proteins depend on the hydrophobic surface properties because the nonpolar residues that are buried in the interior of the molecule should not directly affect these properties.

5. *Accessibility of Residues*

The above considerations have led to attempts to evaluate the surface area of protein molecules that are accessible to the solvent (Lee and Richards, 1971; Chothia, 1976; Janin, 1979). This can be done for proteins whose structures have been elucidated by X-ray crystallography, making the plausible assumption that the solution configuration is the same as that in the crystal. For example, Rose *et al.* (1985) have measured two related quantities, both measures of the hydrophobic contribution: (1) the area that a residue buries upon folding and (2) the fractional accessibility of a residue.

Another approach is to use a probe to measure the solvent-accessible surface. The probe may be a molecule that specifically interacts with hydrophobic areas at the surface, as in hydrophobic interaction chromatography (Greene and Kasarda, 1971). Alternatively, it may be an enthalpy of transfer, measured calorimetrically (Schrier *et al.*, 1986). Use of a molecular probe relies on estimates of the contact area between the protein molecule and the nonpolar surface of the probe. When different probes are used, results can be different, particularly if the protein molecule has a complex geometry.

To avoid this difficulty, Melander and Horvath (1977) proposed a method of estimating surface hydrophobicity based on analysis of solubility data and the effects of neutral salts. The reader is referred to the original paper for the derivation. The advantage of this approach is that the protein molecules themselves act as hydrophobic probes in a salting-out process, giving rise to a natural hydrophobicity scale. Melander and Horvath defined a relative surface hydrophobicity (RSH) as the surface hydrophobicity divided by the molecular weight. Comparison of several proteins showed that neither the average hydrophobicity nor the nonpolar side chain frequency correlated well with the RSH. However, the interesting result emerged that there was an inverse relationship between RSH and CHF. This may be rationalized as follows. Charged groups are supposed

to be exclusively on the surface of the protein molecule. A higher frequency of charged groups is therefore expected to result in a lower frequency of nonpolar groups on the surface. Thus, RSH should decrease as CHF increases.

E. APPLICATION TO WHEAT PROTEINS

Values for the different parameters that have been discussed above are summarized in Table I for wheat glutenin and gliadin, together with corresponding data for three other proteins. Although considerable analytical data are available for wheat proteins, many of the studies do not include data on the degree of amidation. It is therefore not possible to evaluate CHF in those cases. The absence of crystal structure information and molecular weight measurements (on glutenins) prevents complete evaluation of parameters. It appears clear from Table I that gluten proteins are not particularly hydrophobic in comparison to other proteins, as indicated by their NPS, p, and $H\Phi$ values. They are, however, notably deficient in charged groups. From the relationship between CHF and RSH suggested by the three comparison proteins in Table I, very high values for the RSH of the gluten proteins would be predicted. This distinctive feature, together with the assumed very high molecular weights of some glutenins, helps to explain the difficult solubility properties of the gluten proteins in aqueous media.

1. Surface Hydrophobicities

Several methods have been applied for studying the surface hydrophobicities of wheat proteins. These include hydrophobic interaction chromatography (HIC), reversed-phase high-performance liquid chromatography (RP-HPLC), and methods involving interactions with apolar ligands in solution (Popineau and Pineau, 1987). Purified fractions of γ-gliadins have been prepared by Popineau and Pineau (1985) and studied by HIC on phenyl Sepharose CL-4B. From amino acid analysis, the average hydrophobicities of nine fractions ranged from 4.32 to 4.61 kJ per residue. A relationship was observed between protein mobility in two-dimensional polyacrylamide gel electrophoresis (PAGE) and retention by the hydrophobic gel at basic pH. Proteins that were eluted from the gel by the same ethanol concentrations migrated similarly, showing that they carried the same electrical charge. Those proteins with the highest surface hydrophobicities had the lowest apparent contents of charged acidic amino acids, and vice versa, in accordance with the considerations previously discussed in Section II,D,5. The different numbers of charges carried by the fractions appeared to be related to differences in the degree of amidation of the aspartic and glutamic acid residues. There appeared to be a good relationship between the surface

hydrophobicities deduced from the HIC and the average hydrophobicities for these closely related gliadins.

2. *Effects of Neutral Salts*

Following the theoretical ideas developed by Melander and Horvath (1977) and also by Von Hippel and Schleich (1969) and Franks (1978), Preston (1981) studied the solubility properties of gluten proteins in varying concentrations of the so-called lyotropic (or Hofmeister) series of neutral monovalent sodium salts. The use of these salts is based on the premise that, at sufficiently high salt concentrations, electrostatic interactions are minimized and water structure is perturbed. Solubility and other properties of proteins resulting from variations in the concentrations and nature of anions of the lyotropic series are then directly related to hydrophobic effects. Large variations in solubility were observed as the anion was varied along the series in the order F^-, Cl^-, Br^-, ClO_4^-, I^-, and SCN^-. These effects are illustrated in Fig. 3 for the two extreme ions in the series, and the results for all the salts at a fixed concentration of 1.0 *M* are summarized in Table II. There is a spectacular contrast in effects between the different anions (Fig. 3), confirming that hydrophobic interactions are of prime importance in determining the solubility of gluten proteins. The effects of the different anions are believed to be related to the ordering of water structure, this

TABLE II

EFFECTS OF NEUTRAL SODIUM SALTS (1.0 *M*) ON THE SOLUBILITY (%) OF DEFATTED AND NONDEFATTED GLUTEN[a]

	Gluten	
Salt	Defatted	Nondefatted
None (H_2O)	25.0	29.5
NaF	Trace	Trace
NaCl	5.8	5.2
$NaBrO_3$	5.4	6.4
NaBr	19.9	Not determined
$NaClO_4$	31.3	28.7
NaI	51.7	54.6
NaSCN	61.5	59.1
HAc (0.05 *M*)	70.4	77.2
Lactic (0.005 *M*)	71.4	75.8

[a]From Preston (1981).

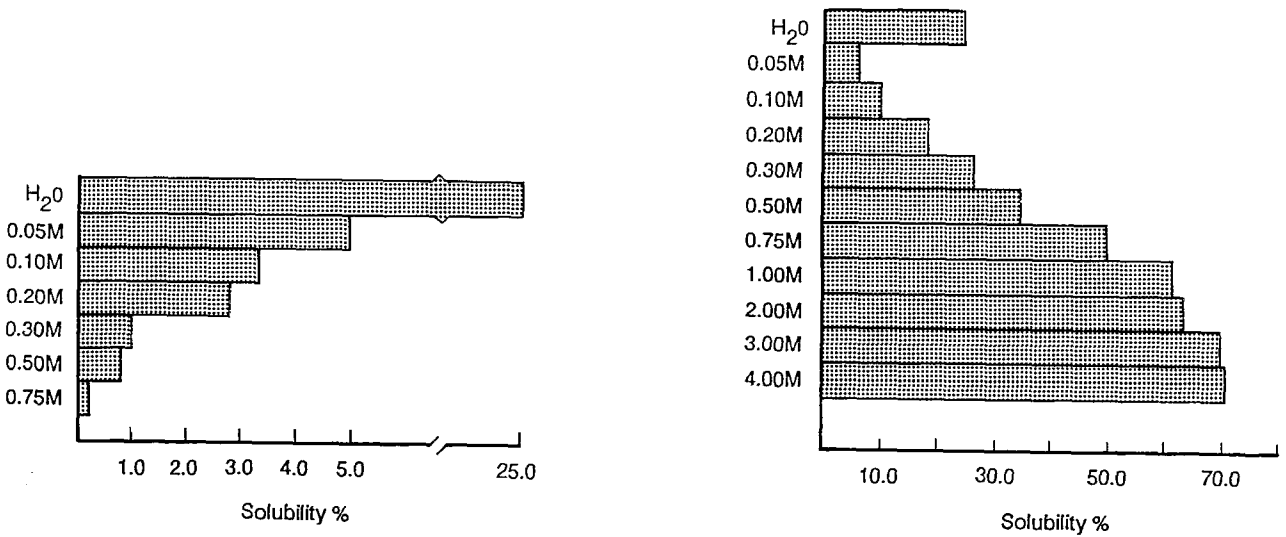


FIG. 3. Effects of increasing concentrations of salts on the solubility of Neepawa defatted gluten; left, effects of sodium fluoride; right, effects of sodium thiocyanate (Preston, 1981).

ordering being highly dependent on the ion type. Nonchaotropic ions such s F^- and Cl^- strongly promote ordering whereas ions such as I^- and SCN^- at the other end of the series are chaotropic (structure breaking) and have only small ordering effects. These differences are thought to be the basis of the lyotropic (Hofmeister) series. The net result is that for the chaotropic ions such as SCN^-, the negative entropy changes associated with exposure of nonpolar amino acid residues to the aqueous solvent are much reduced. Interaction between nonpolar groups and water molecules thus becomes more favorable thermodynamically, accounting for the higher solubility of gluten proteins.

F. SOLUBILIZATION OF HIGHEST MOLECULAR WEIGHT GLUTENIN

The solubility of proteins is hindered by hydrophobic effects resulting from the high content of nonpolar side chains. This effect is usually minimized by folding of molecules so as to bury a large proportion of the nonpolar side chains in the interior of the molecule, where they are shielded, at least partially, from interaction with water molecules. However, gluten proteins, because of their low CHF, are thought to have high RSH. A number of approaches can be used to increase gluten protein solubility, namely, increasing temperature (MacRitchie, 1973), using chaotropic salts (Preston, 1981), and conferring a net charge by altering the pH or by using ionic surfactants. Although solubilization of a large proportion of the protein can be achieved by all these methods, a significant amount (of the order of one-third) of the glutenin fraction usually remains undissolved and this amount varies from one wheat variety to another. This is unsatisfactory when a comparison of the protein compositions of different varieties is required. The protein not solubilized is believed to be the highest molecular weight glutenin. If reducing agents are used to break interchain disulfide bonds, complete solubilization can be achieved, but information about the native glutenin structure is then lost. The failure of the native glutenin to dissolve does not appear to be due to a compositional effect, as glutenin and gliadin compositions are quite similar. Evidently, the small decrease in free energy resulting from the entropy of mixing is insufficient to compensate for the other positive free energy changes for these large molecules with all solvents that have been used.

1. Increase of Solubility by Dough Mixing

An interesting method for increasing the solubility of wheat protein was discovered through analysis of dough mixing (Mecham, 1968; Tsen, 1969; Tanaka and Bushuk, 1973a). In these studies, it was observed that the extra protein that

was solubilized was derived from the glutenin size range. The behavior was similar to what is observed with high polymers when they are subjected to shear stress, as may occur in high-speed mixing. The increased solubility arises as a result of scission of the largest polymer molecules. Scission occurs because molecules cannot disentangle rapidly enough in response to the shear stress. According to the theory for this process, the greatest tension occurs at the centers of molecules, and as a consequence, molecules break preferentially near their centers.

A theory for chain scission of polymers in shear has been proposed by Bueche (1960). According to this theory, the probability that a particular chain of molecular weight M in a sample of average molecular weight M_t will break at its center is given by

$$P\,dt = K \exp -(E - F_0\delta)/kT\,dt \tag{3}$$

where E is the energy required to break a chain bond, K is a constant whose magnitude is approximately equal to the bond vibration frequency, and δ is approximately half the length of a chain bond. The term F_0 is given by

$$F_0 = (\text{constant})\dot{\gamma}\eta/\rho(M/M_t)^2 \tag{4}$$

where the constant has a value of approximately 10^{-13} cgs units (providing a coiling polymer with many entanglements is being considered), $\dot{\gamma}$ is the shear strain rate, η is the viscosity, and ρ is the density.

Substitution of plausible values for the terms in Eq. (2) has shown that the strain rates produced in high-speed mixing are sufficient to cause molecular scission (MacRitchie, 1975). Equation (2) also predicts that, for a polymer with a wide molecular weight distribution, only those molecules with a molecular weight above a certain critical value, M_{max}, are broken. Because molecules break preferentially near their centers, the products of scission (of molecular weight M fall within the narrow molecular weight range of $M_{max} > M > M_{max/3}$. The theory appears to agree with the observations made for the effects of dough mixing. The highest molecular weight glutenin is broken down into proteins that are still large enough to fall within the glutenin size range.

2. *Use of Ultrasound (Sonication)*

Other methods are available for controlled reduction in the size of large glutenin molecules, permitting their solubilization. These include high-speed stirring or use of ultrasound (or sonication) to mix suspensions. Sonication has been found to be particularly convenient (Singh and MacRitchie, 1989). The effect of increasing the sonication time for a dilute wheat flour suspension in SDS solution

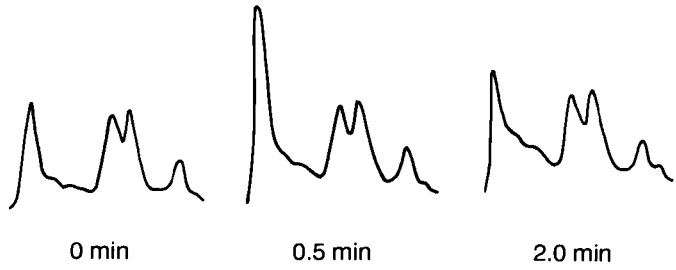


FIG. 4. Effects of increasing sonication time of a flour suspension on the SE-HPLC profile of the solubilized protein (Singh and MacRitchie, 1989).

is shown in Fig. 4, where the change in the protein size distribution has been monitored by size-exclusion high-performance liquid chromatography (SE-HPLC). Initially, there is a sharp increase in the height of the first peak, which corresponds to glutenin. As sonication proceeds, there is a shift in the size distribution of the glutenin as seen by the decrease in height of the first peak and an increase

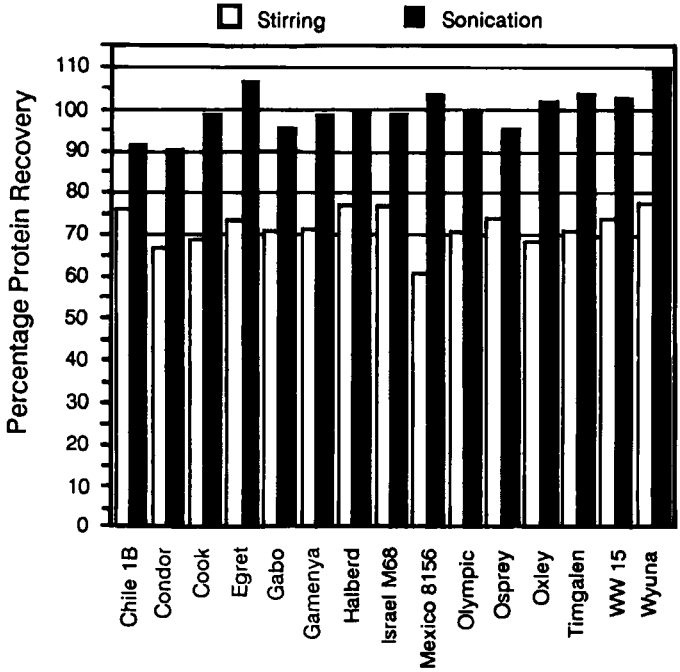


FIG. 5. Extractability of flour protein (based on bicinchoninic acid determination on the extracts) from 15 wheat varieties without and with sonication (Singh *et al.*, 1990b).

in the height of the shoulder on the trailing edge. The aim in using sonication is to optimize the time so as to solubilize the maximum amount of protein without altering the SE-HPLC profile.

A comparison of the proportions of protein extracted from flour samples of 15 wheat varieties with and without sonication is shown in Fig. 5 (Singh *et al.*, 1990b). The average protein extractability (percentage of the total protein extracted) without sonication was 72.1% (with a range of 60.6–78% and a coefficient of variation of 6.5). A significant negative correlation ($r = -0.75$, $P = 0.0013$) was found between protein extractability and extensigraph maximum resistance (R_{max}), a measure of dough strength. After optimized sonication, protein extractability averaged 100% (with a range of 90.4–109.8% and a coefficient of variation of 5.2) and showed no significant correlation with R_{max}. An extraction of at least 95% of the flour protein has been reported for the method (Singh and MacRitchie, 1989; Batey *et al.*, 1991). These results indicate that close to complete solubilization of flour protein is achieved using sonication. The negative correlation observed between amount of solubilized protein and R_{max} therefore points to the danger in drawing conclusions based on measurements of partially solubilized protein from a range of wheat varieties.

III. CLASSIFICATION

A. SOLUBILITY-BASED CLASSIFICATIONS

The earliest classification of cereal proteins is based on a fractionation procedure introduced by Osborne (1907). This uses sequential extraction to separate albumin/globulins (soluble in salt solution), prolamins (70% aqueous ethanol soluble), and glutelins (insoluble in either salt or 70% ethanol). In the case of wheat, the prolamins correspond to gliadins and the glutelins to glutenins. Minor modifications of this classical method have been used since its introduction, but, even today, the Osborne procedure is considered to be a sound basis for the separation of wheat proteins into their main groups. When, however, accurate quantitative measurements are required, solubility fractionation is not entirely satisfactory because of the overlap between the different fractions. This is illustrated in Fig. 6, where SE-HPLC profiles for each of the three main Osborne fractions are compared with that of the total protein. Other problems with the Osborne classification arise when conditions such as the type of alcohol used, the composition of the extractant, and the temperature are varied, leading to different extractability and to differing polypeptide composition of the solubilized material (Byers *et al.*, 1983).

The amino acid compositions of the prolamins and glutelins of wheat are reasonably similar (see Table III). Prolamins (gliadins) certainly have more

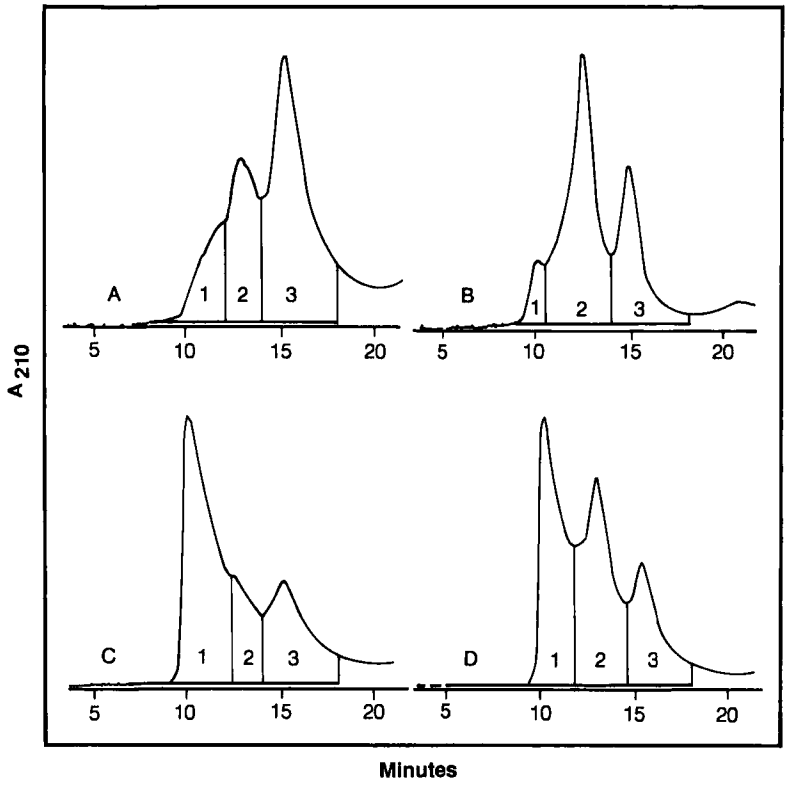


FIG. 6. SE-HPLC chromatograms of total protein extract and solubility fractions from Mexico 8156 flour. (A) Albumin/globulin (0.05 *M* NaCl extract); (B) gliadin (70% ethanol extract); (C) glutenin (residue after salt and ethanol extractions); (D) total protein extract. Regions 1, 2, and 3 of the chromatograms represent polymeric proteins, gliadins, and albumin/globulins, respectively. Total protein and the glutenin fraction were extracted using sonication in SDS–buffer solution (Singh *et al.*, 1990a).

proline and less glycine than do glutelins (glutenins). However, the main difference between them is that the prolamins consist of single polypeptide chains whereas the glutelins are made up of multiple chains bonded together by disulfide bonds. The resulting high molecular weight of the glutelins is responsible for their comparative insolubility, as discussed in Section II. Shewry *et al.* (1986) revised the Osborne classification by noting that, when the disulfide bonds of the polymeric glutelins are broken, their individual chains (usually termed subunits) become soluble in aqueous alcohol. They therefore argued that they should also be considered as prolamins. There is good justification for this nomenclature because of the similarity in composition of all these proteins. This similarity, in turn, is thought to be a consequence of their common genetic ancestry, another

TABLE III

AMINO ACID COMPOSITION OF GLUTENINS AND GLIADINS[a]

	Anhydro amino acid (g) in 100 g of recovered anhydro amino acids		Amino acid residues in 10^5 g of recovered anhydro amino acids	
Amino acid	Glutenin	Gliadin	Glutenin	Gliadin
Cys (half)	2.37	2.97	23.2	29.0
Met	1.66	1.43	12.6	10.9
Asp	3.73	2.86	32.5	24.8
Thr	3.11	2.16	30.7	21.3
Ser	5.38	4.67	61.8	53.6
Glu	33.15	38.87	256.8	301.1
Pro	10.29	13.79	105.9	142.0
Gly	3.82	1.53	67.0	26.8
Ala	2.77	2.03	39.0	28.6
Val	4.24	4.12	42.8	41.6
Ile	3.74	4.31	33.0	38.0
Leu	6.58	6.85	58.1	60.6
Tyr	3.61	2.61	22.1	16.0
Phe	4.76	5.52	32.4	37.5
Lys	2.26	0.64	17.6	5.0
His	2.32	2.23	16.9	16.3
Arg	4.13	2.72	26.5	17.4
Trp	2.09	0.71	11.2	3.8
Amide N	3.31	4.19	236.1	298.7
(Amide × 100)/(Glu + Asp)			81.7	91.7

[a]Means from four varieties. From Ewart (1967).

reason for classifying them together. Three main groups of prolamins were defined—sulfur-rich, sulfur-poor, and high-molecular-weight prolamins.

On the other hand, Ewart (1990) has strongly criticized the basis of the classification by Shewry *et al.* (1986), arguing that the polymeric glutenins are fundamentally different from the monomeric gliadins because of their intermolecular disulfide bonding capacity. This property of the glutenins results in contributions to functionality that are very different from those of the gliadins. There is a certain amount of validity in each of the two opinions, and whichever is adopted will partly depend on the particular approach being followed. In this article, we will be considering both the genes that control the synthesis of the different wheat proteins and the contributions of the different proteins to functionality. Therefore, we will be concerned with the proteins at the subunit (gene-related) level as well as with their native (functionally related) structures.

B. MOLECULAR-SIZE-BASED CLASSIFICATIONS

As discussed in Section II, the difficult problem of solubilizing the total wheat protein can be largely overcome by using sonication of a flour dispersion in SDS–buffer solution. The relative amounts of the main protein classes may then be determined using SE-HPLC. A typical chromatogram is shown in Fig. 7B, with an eluting solvent of acetonitrile/water + 0.1% trifluoracetic acid (TFA).

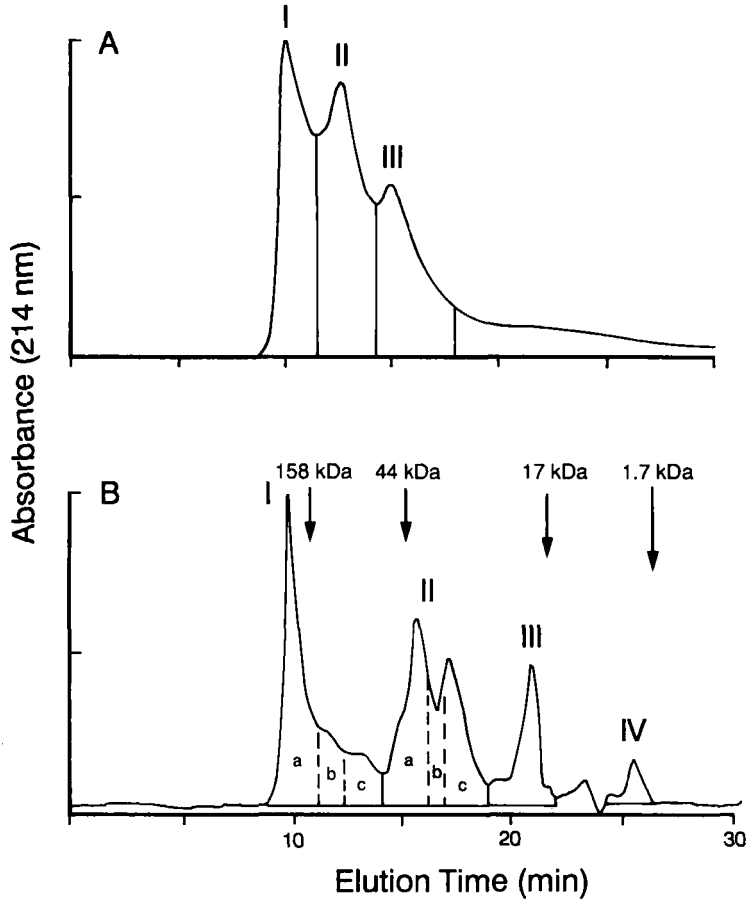


FIG. 7. SE-HPLC profiles of total protein from Cook flour extracted in SDS–buffer solution using sonication. (A) Profile obtained using 0.1% SDS–buffer solution as eluting solution. (B) Profile obtained using 50% acetonitrile and water containing 0.1% trifluoroacetic acid. The dashed lines show the cutoff points for collection of fractions that were used to obtain the SDS–PAGE patterns shown in Fig. 8. The elution times of molecular weight markers are shown by arrows; peak I was at the void volume (Batey *et al.*, 1991).

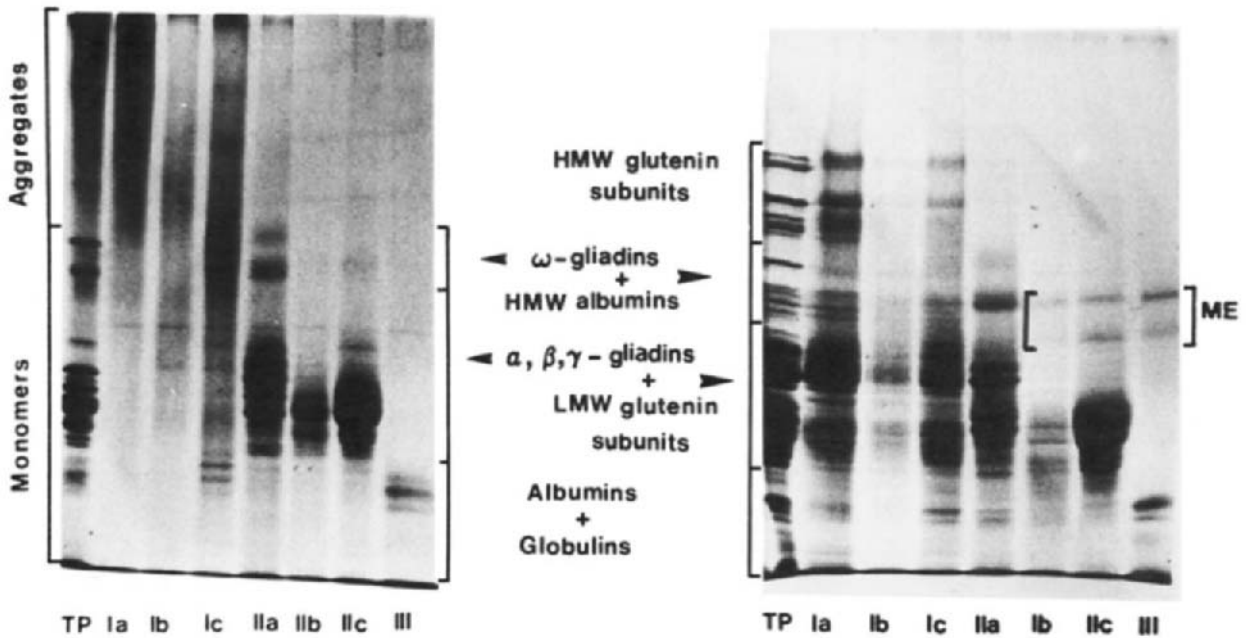


FIG. 8. One-dimensional SDS–PAGE patterns of flour proteins and fractions from the Cook flour in Fig. 7B under unreduced (left) and reduced (right) conditions. TP, Total proteins; Ia–Ic, peak I proteins; IIa–IIc, peak II proteins; III, peak III proteins; ME, mercaptoethanol (Batey *et al.*, 1991).

The protein is separated into three main groups. By collecting fractions and running SDS–polyacrylamide gel electrophoresis (SDS–PAGE), it has been established that these three groups, in order of elution as shown in Fig. 7, correspond to (I) polymeric (i.e., intermolecular disulfide-bonded) proteins, (II) gliadins, and (III) monomeric albumin/globulins. The SDS–PAGE patterns of fractions collected from the SE-HPLC column are shown in Fig. 8.

In attempting to relate functional properties or behavior to protein composition, a size-based classification appears to be superior to a solubility-based one. Because of the heterogeneity of wheat proteins, it is difficult to effect a sharp separation using differential solubility. For example, aqueous ethanol extracts a certain amount of polymeric proteins as well as the gliadins. These have at times been referred to as HMW gliadins (Bietz and Wall, 1980). Here, the solubility classification breaks down, because these proteins are disulfide bonded and are more correctly designated as glutenins, albeit probably constituting the lowest molecular size polymers. Of course, in a technique such as SE-HPLC, a certain amount of overlap may also occur between fractions. However, this is an experimental limitation. In theory, it should be possible to effect a sharp separation, whereas this is not necessarily the case with solubility. We will now consider in more detail the makeup of the proteins in each of the size-exclusion fractions.

C. POLYMERIC PROTEINS

Three main types of proteins eluting in peak I of the size-exclusion chromatogram (Fig. 7) have been identified as glutenins, high-molecular-weight (HMW) albumins (mostly β-amylases), and triticins (or HMW globulins).

1. Glutenins

The main component of peak I protein is glutenin. As mentioned, this protein consists of a heterogeneous mixture of polymers formed by disulfide-bonded linkage of three main size groups of polypeptides or subunits. Glutenins in their native state are not resolved by gel electrophoresis. The large molecules either do not enter the gel or they form a smear near the origin. However, groups of subunits are identified by SDS–PAGE after reduction of glutenins by a disulfide bond-breaking reagent such as mercaptoethanol (ME) or dithiothreitol (DTT). When the total reduced protein is loaded on the gel, one set of glutenin subunits is clearly separated as the components showing lowest mobility (Fig. 9B). These have been called the high-molecular-weight (or A) glutenin subunits. They are obviously the largest components in the reduced protein mixture. Their molecular weights (M_r) from SDS–PAGE range from about 80,000 to 120,000, although other estimates, such as obtained from amino acid sequencing, give a rather lower range of 60,000–90,000 (Anderson *et al.*, 1988). The other two groups

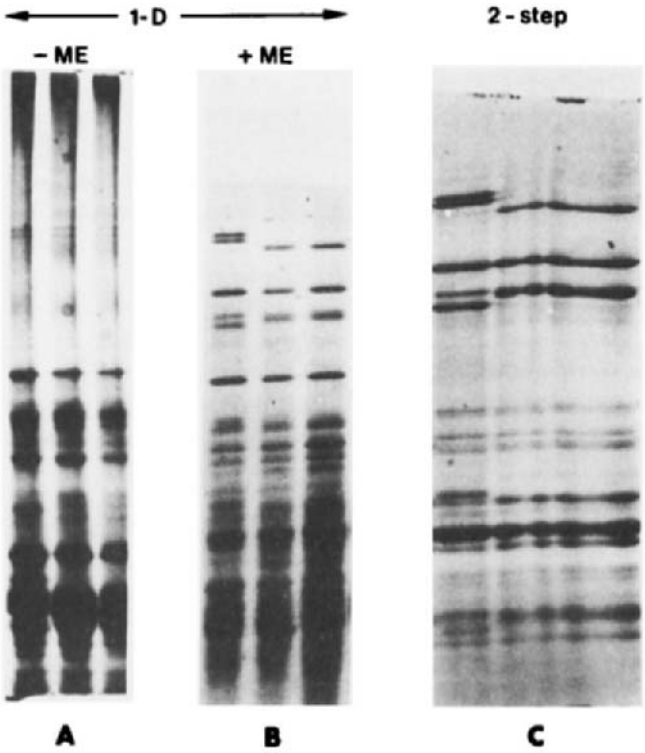


FIG. 9. One-dimensional SDS–PAGE illustrating a two-step procedure for separating glutenin subunits from monomeric proteins. (A) Unreduced total proteins; (B) reduced total proteins; (C) reduced polymeric proteins after separation from monomeric proteins; ME, mercaptoethanol (MacRitchie *et al.*, 1989).

of subunits have mobilities similar to many of the gliadin components and therefore their electrophoretic bands overlap with those of gliadins. In order to resolve them, some way of separating polymeric from monomeric components needs to be found. One method that has been used is a two-step procedure (Singh and Shepherd, 1988) in which the unreduced total protein is first loaded on the gel. The gliadins and other monomeric components are allowed to run ahead of the polymeric components, which remain in the starting slot or form a smear near the origin. The protein from the slot and the smear is then removed and run under reducing conditions on SDS–PAGE. The result of this second step is shown in Fig. 9C. This shows the slowest mobility A subunit bands and below them the other two sets of faster mobility bands due to the B and C glutenin subunits. The B and C subunits together are usually referred to as the low-molecular-weight (LMW) glutenin subunits. An alternative two-step procedure

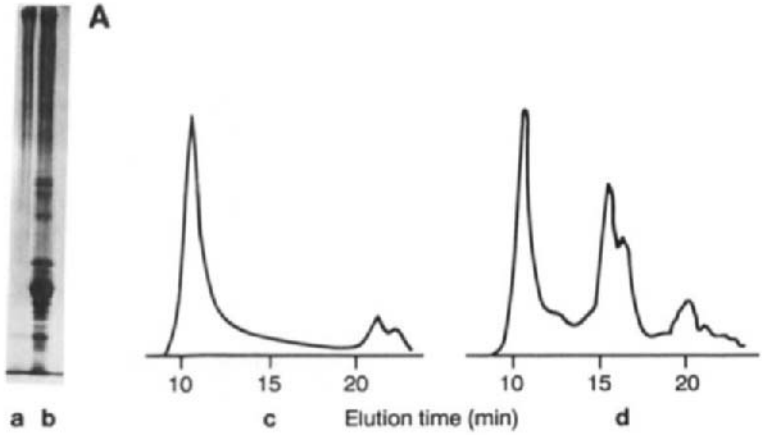
is to collect the peak I fraction from SE-HPLC of wheat protein. The second step is then the same as in the previous method. A more rapid procedure that avoids the first electrophoretic step consists in first removing gliadins by solubilization in dimethyl sulfoxide (DMSO) (Gupta and MacRitchie, 1991). Results from this procedure are shown in Fig. 10.

Another method that shows much promise for quantitative measurement of the proportions of HMW and LMW glutenin subunits is reversed-phase HPLC. Until now, this method has been mainly used to estimate HMW subunits (Marchylo *et al.*, 1989; Sutton *et al.*, 1989; Seilmeir *et al.*, 1991), but the technique is being refined to measure both HMW and LMW sets of subunits (K. H. Sutton, personal communication). From SDS–PAGE, the molecular weights of B subunits range from 40,000–55,000 and C subunits from 30,000–40,000. It has been estimated that the ratio of LMW to HMW subunits in glutenin is roughly three to one for normal hexaploid wheats, although this ratio varies with genotype (Gupta *et al.*, 1992).

2. *Triticins (HMW Globulins)*

SDS–PAGE of unreduced total protein extracts from hexaploid wheat endosperm shows three slow-moving bands (denoted triplet bands) in a zone of heavy background streaking. The proteins associated with these bands have been studied in detail by Singh and Shepherd (1985) and have been named triticins. The amino acid compositions and solubility properties of these proteins resemble those of rice glutelin and the storage globulins of oats and legumes. By using a nonreducing/reducing form of two-dimensional electrophoresis, the triticin proteins were shown to be disulfide linked and to be heterotetramers made up of four subunits. These subunits have been designated as D (M_r 58,000), δ (M_r 22,000), A (M_r 52,000), and α (M_r 23,000). The structure of the triticins is depicted in Fig. 11. The three triplet bands in decreasing order of size correspond to T1 (DδDδ), T2 (DδAα), and T3 (AαAα). On partial reduction, the tetramers dissociate into dimers and, on further reduction, are reduced to monomers, as shown in Fig. 11. In SE-HPLC, the triticins are among those proteins eluting in peak I that have the longest retention times and therefore lowest molecular weights (see Fig. 8).

FIG. 10. (A) One-dimensional one-step electrophoretic procedure for separation of polymeric proteins. (a) SDS–PAGE of unreduced protein from residue after extraction with DMSO (polymeric protein); (b) SDS–PAGE of unreduced total protein; (c) SE-HPLC of residue (same fraction as used in a); (d) SE-HPLC of total protein as used in b. (B) One-dimensional SDS–PAGE separation of HMW and LMW glutenin subunits showing comparison between one-step (left) and two-step (right) procedures. Positions of the M_r markers are shown on the left side of the figure and names of varieties at the bottom (Gupta and MacRitchie, 1991).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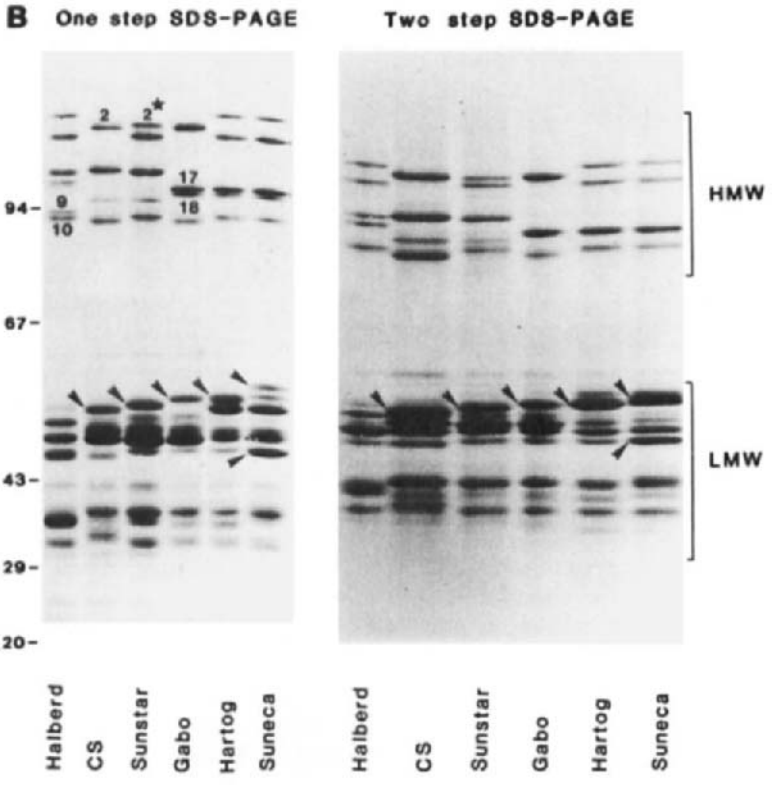
B One step SDS-PAGE
Two step SDS-PAGE
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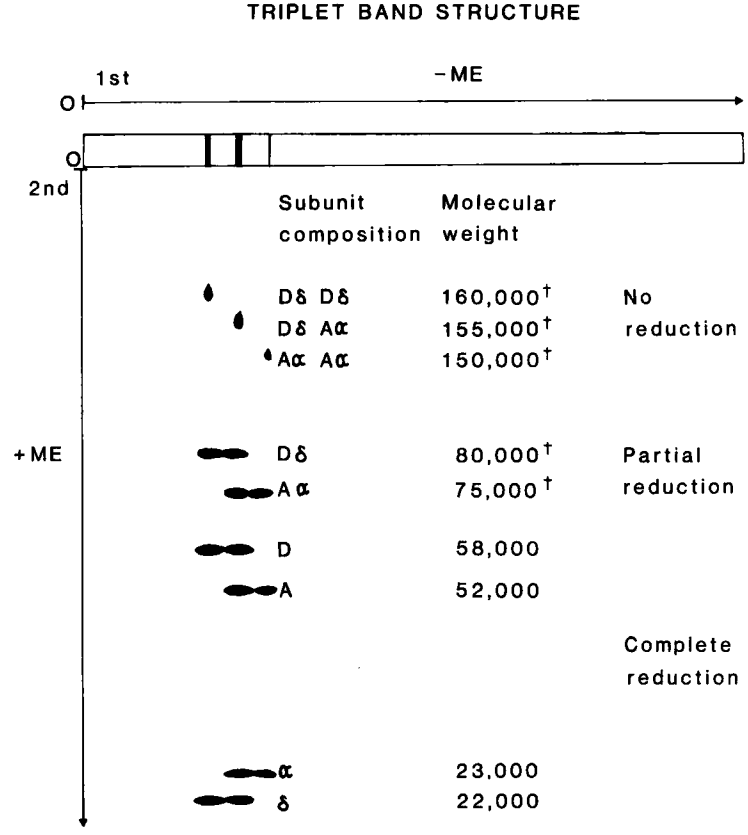


FIG. 11. Subunit composition of triticins as indicated by a nonreducing/reducing two-dimensional electrophoresis procedure (Singh and Shepherd, 1985).

3. *HMW Albumins*

Another group of bands (69, 63, 60, and 45 kDa) appears in the SDS–PAGE pattern of peak I protein after reduction of disulfide bonds (e.g., by mercaptoethanol). The proteins corresponding to these bands are insoluble in aqueous ethanol, showing them to be nonprolamins. However, they are soluble in water and can be classed as albumins (Gupta *et al.*, 1991c). Their elution with polymeric glutenins and triticins in peak I of SE-HPLC suggests that they are also disulfide-linked molecules. This has been confirmed by applying diagonal electrophoresis (unreduced first dimension, reduced second dimension), as shown in Fig. 12. With this procedure, bands that form parallel lines or spots away from the diagonal are considered to be disulfide-linked polymers in the native

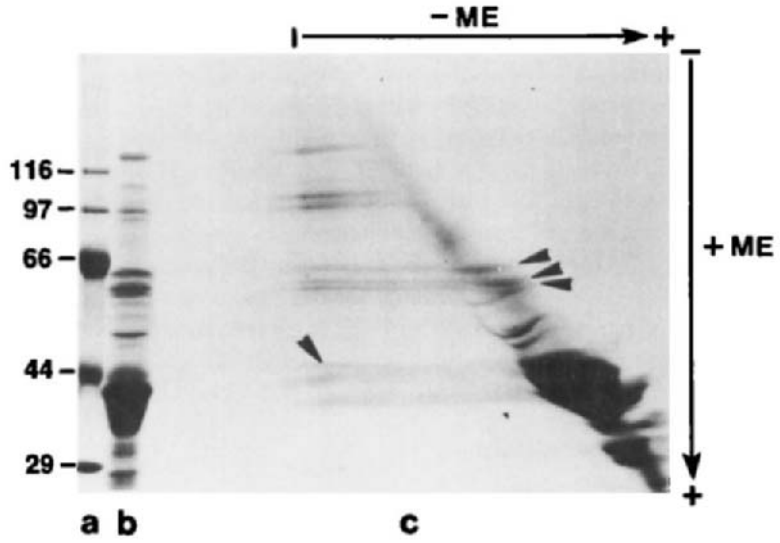


FIG. 12. One-dimensional SDS–PAGE of (a) M_r standards, (b) reduced total proteins from triple rye translocation (TTr) stock, and (c) diagonal (unreduced × reduced) electrophoresis of total proteins from the TTr stock. HMW albumin bands are indicated by arrowheads (Gupta *et al.*, 1991c).

state. Spots on the diagonal correspond to monomers. The albumin bands (Fig. 12) form both parallel lines as well as spots on the diagonal, indicating that they occur in both polymeric and monomeric forms in their native state. There is evidence that a large proportion of the albumin subunits is mutually linked by disulfide bonds and not linked to glutenin subunits. For example, they appeared as parallel lines in two-dimensional gels even when most of the glutenin and all the triticin subunits were absent (Fig. 12). Furthermore, they were absent from preparations of native glutenin (Tao *et al.*, 1989) when extracted in 0.1 *M* acetic acid. However, since only 70% of the protein was extracted by acetic acid, the possibility that heteropolymers of glutenin and albumin exist cannot be ruled out. Immunological analysis of HMW albumins has confirmed that the 69-, 63-, and 60-kDa proteins are β-amylases, but not the 45-kDa protein (Gupta *et al.*, 1991c).

D. MONOMERIC PROTEINS

1. Gliadins

Peak II of the SE-HPLC profile of Fig. 7 corresponds to gliadins. Four main groups of gliadins are usually distinguished in electrophoresis. These are α, β, γ, and ω in decreasing order of mobility and therefore increasing molecular size.

This nomenclature was originally based on separation of bands according to mobility on starch gels at pH 3 (Woychik *et al.*, 1961), and this has proved to be a useful classification. The ω-gliadins, the highest molecular weight group, are the sulfur-poor prolamins of Shewry *et al.* (1986). They are clearly separated from other gliadins in SDS electrophoresis (see Fig. 13), whereas there is often some overlap of the α-, β-, and γ-gliadins. Gliadins do have disulfide bonds, but all are apparently intramolecular. All gliadins are soluble in aqueous ethanol in their native state and are thus true prolamins.

The nomenclature for gliadins has been critically discussed by Bushuk and Sapirstein (1991) and a more precise definition has been proposed. This is based on their relative electrophoretic mobility using three reference bands (Sapirstein and Bushuk 1985). Relative mobilities of the four gliadin groups according to this nomenclature are ω (<40.4), γ (40.4–53.2), β (53.2–68.6), and α (>68.6) (Fig. 13).

2. *Albumins/Globulins*

Proteins eluting in peak III of the SE-HPLC profile (Fig. 7) are a mixture of low-molecular-weight compounds, many of which are enzymes. They are usually called albumins (water soluble) and globulins (salt soluble), in line with the Osborne classification. Like the HMW albumins and triticins, the amino acid composition is quite different from that of the gluten proteins, glutenin and gliadin (see Table IV). Their insolubility in aqueous ethanol places them in the nonprolamin class.

IV. GENETIC CONTROL

Consideration of desirable physicochemical properties of wheat proteins, such as appropriate viscoelasticity, is one general objective of breeding varieties having optimum end-use characteristics. One of the areas wherein recent scientific progress has had the greatest impact in relation to this problem has been in identifying the chromosomal location of genes controlling the synthesis of different wheat proteins. It is therefore appropriate to summarize this knowledge, as this will be highly relevant when considering strategies for manipulating protein composition to target specific end-use requirements. In summarizing this information, the same order for consideration of the protein groups will be used as was followed for their classification in Section III. This is because it divides the proteins more clearly into groups having similar effects on functionality, and it thus provides an appropriate basis to discuss breeding strategies (Section VIII).

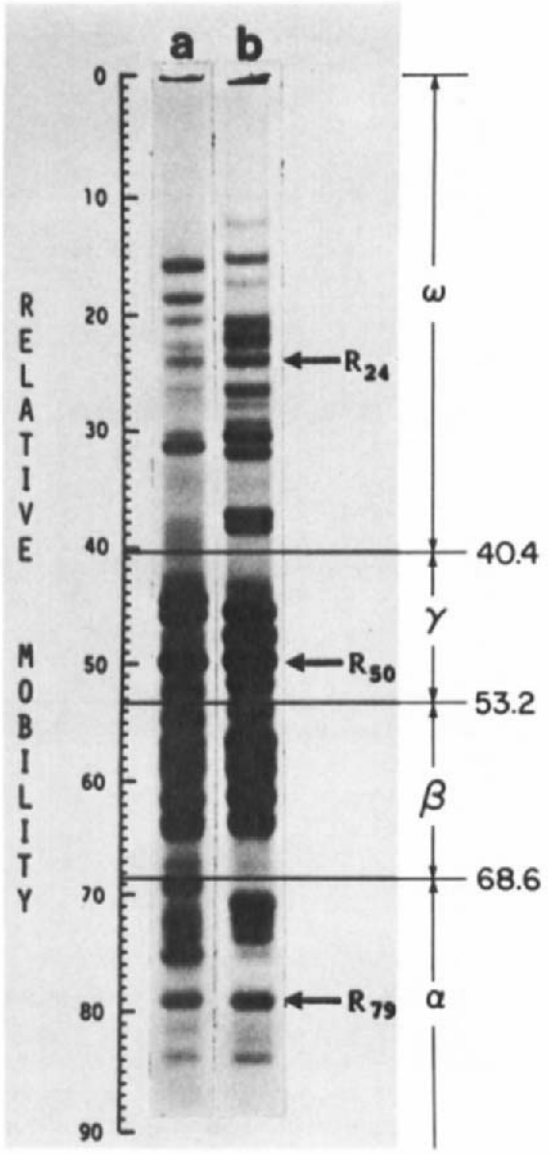


FIG. 13. PAGE electrophoregrams of gliadins from Canadian wheat cultivars Marquis (a) and Neepawa (b) showing the four main groups of gliadins and the proposed boundaries between them based on three reference (R) bands (Bushuk and Sapirstein, 1991).

TABLE IV

AMINO ACID COMPOSITION OF DIFFERENT WHEAT PROTEINS[a]

Amino acid	Glutenin HMW subunits[b]	Gliadins			Triticin A + D subunits[f]	β-Amylase[g]	Albumin (LMW)[h]
		α-1B[c]	γ-1[d]	ω[e]			
Asp	2.8	2.8	2.9	0.5	7.2	11.0	7.6
Thr	3.2	1.5	1.9	1.1	3.0	3.1	2.4
Ser	6.4	5.5	5.4	3.4	7.1	3.9	6.4
Glu	32.6	38.4	39.9	50.1	20.3	11.1	10.8
Pro	12.6	14.6	15.3	22.9	4.9	6.1	7.5
Gly	8.8	2.5	2.4	1.6	16.2	8.8	8.3
Ala	5.0	2.5	3.4	0.6	5.8	8.0	8.4
Val	3.5	4.7	4.4	0.5	4.3	7.3	11.3
Cys (half)	0.8	2.2	2.1	0.4	1.0	1.6	8.1
Met	0.7	0.7	0.8	0.1	0.8	2.4	2.6
Ile	1.7	4.4	4.7	3.7	2.9	3.9	1.7
Leu	5.3	8.5	7.1	3.4	6.0	8.7	7.6
Tyr	4.4	3.2	2.8	1.0	3.0	4.5	3.4
Phe	1.6	3.6	3.7	9.1	5.3	4.4	0.1
Lys	0.9	0.6	Trace	0.9	2.8	3.8	5.0
His	1.4	2.0	1.5	0.4	3.2	3.7	0.02
Arg	1.8	1.9	1.4	0.6	6.2	5.4	5.7
Trp	n.d.	0.4	0.2	n.d.	n.d.	2.5	3.0

[a]Values given in mol %; n.d., not determined.
[b]Miflin *et al.* (1983).
[c]Platt and Kasarda (1971).
[d]Huebner *et al.* (1967).
[e]Charbonnier (1974).
[f]Singh *et al.* (1991).
[g]Tkachuk and Tipples (1966).
[h]Ewart (1969).

A. CHROMOSOMAL STRUCTURE OF WHEAT

Wheats that have been developed for breadmaking are hexaploids (i.e., *Triticum aestivum*). That is to say, they have three genomes, each with seven pairs of chromosomes, making a total of 42 chromosomes as shown in Fig. 14. The genomes are denoted by the letters A, B, and D and the seven pairs of chromosomes in each genome are numbered from 1 to 7. Each chromosome consists of a long arm and a short arm joined by a centromere (see Fig. 15). Tetraploid wheats have two genomes (A and B) and thus 28 chromosomes. An example is *Triticum turgidum* var. *durum*, used extensively in pasta production. Diploids with one genome and therefore 14 chromosomes are the primitive forms of

Chromosome number in each genome	Genome A	B	D
1	1A	1B	1D
2	2A	2B	2D
3	3A	3B	3D
4	4A	4B	4D
5	5A	5B	5D
6	6A	6B	6D
7	7A	7B	7D

FIG. 14. Chromosome structure of hexaploid wheat.

wheat. In general, they are little utilized commercially but are important in breeding for introducing useful genes into cultivated wheats. In order to simplify discussion, we are going to concentrate on hexaploid wheats. The allocation of genes coding for particular proteins has been achieved by use of genetic variants in combination with electrophoresis. Examples of these variants are aneuploids, lines that are deficient in a single chromosome. Much of the pioneering work

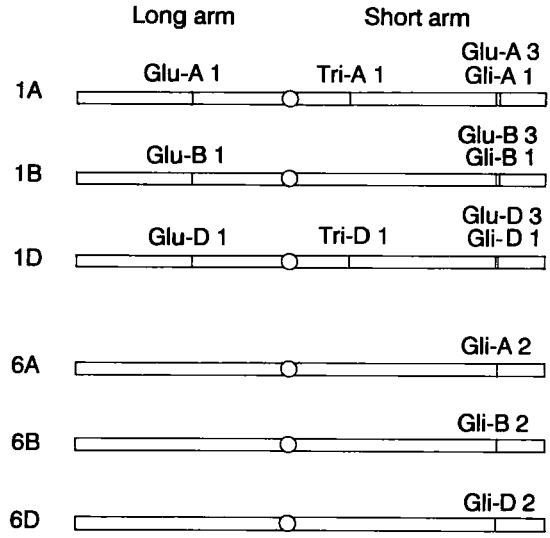


FIG. 15. Chromosomal location of major protein groups of hexaploid wheats.

in this area was conducted by Sears (1969). In simple terms, when a particular chromosome is missing, this may coincide with the disappearance of a certain band or bands in the electrophoretic pattern of the wheat protein, thus identifying the chromosome that carries the gene(s) responsible for the synthesis of the missing protein(s). Using these variants and others, such as substitution or ditelocentric lines, the same principle can be adopted to match proteins with chromosomes or chromosome arms. Furthermore, the positions of the genes controlling specific proteins can be mapped; e.g., using telocentric lines, the distances of the genes from the centromere can be determined where the centromere is identified by biochemical markers on the opposite arm of the chromosome.

B. POLYMERIC PROTEINS

1. HMW (A) Glutenin Subunits

The HMW glutenin subunits are coded by genes (loci) on the long arms of chromosomes 1A, 1B, and 1D as shown in Fig. 15. These loci are designated *Glu-A1*, *Glu-B1*, and *Glu-D1*, respectively. Electrophoretic studies have revealed appreciable polymorphism in the number and mobility of the HMW subunits in different wheat cultivars. That is, the genes on the chromosome 1 long arms show multiple allelism. The different subunits have been studied for a large number of varieties (see, e.g., Lawrence and Shepherd, 1980; Galili and Feldman, 1985) and are nowadays catalogued by the nomenclature introduced by Payne and Lawrence (1983). Based on electrophoretic studies, Payne *et al.* (1981) proposed that there were two main types of subunits, the x type (of high relative molecular weight, M_r) and the y type (low M_r). This subdivision has been supported by chemical and genetic evidence. A particular subunit is specified by noting its chromosome, followed by its classification as x or y, and finally a number (designating the protein subunit), this number increasing with decreasing M_r. Thus Chinese Spring, the variety on which most genetic studies have been based, has the HMW gluten subunit composition 1Bx7, 1By8, 1Dx2, 1Dy12 (being null at the *Glu-A1* locus). Some examples of the allelic variation in HMW subunits of hexaploid wheats are shown in Fig. 16. The maximum number of HMW subunits donated by a single group 1 chromosome is two, evidently corresponding to two genes. However, it is found that the y subunits are never expressed as the *Glu-A1* locus and sometimes not expressed at the *Glu-B1* locus in common hexaploids; i.e., the genes coding for the y subunits are silent in these cases. In certain diploid wheats, on the other hand (e.g., *Triticum thaoudar*, equivalent to the A genome of hexaploids), both x and y subunits are expressed.

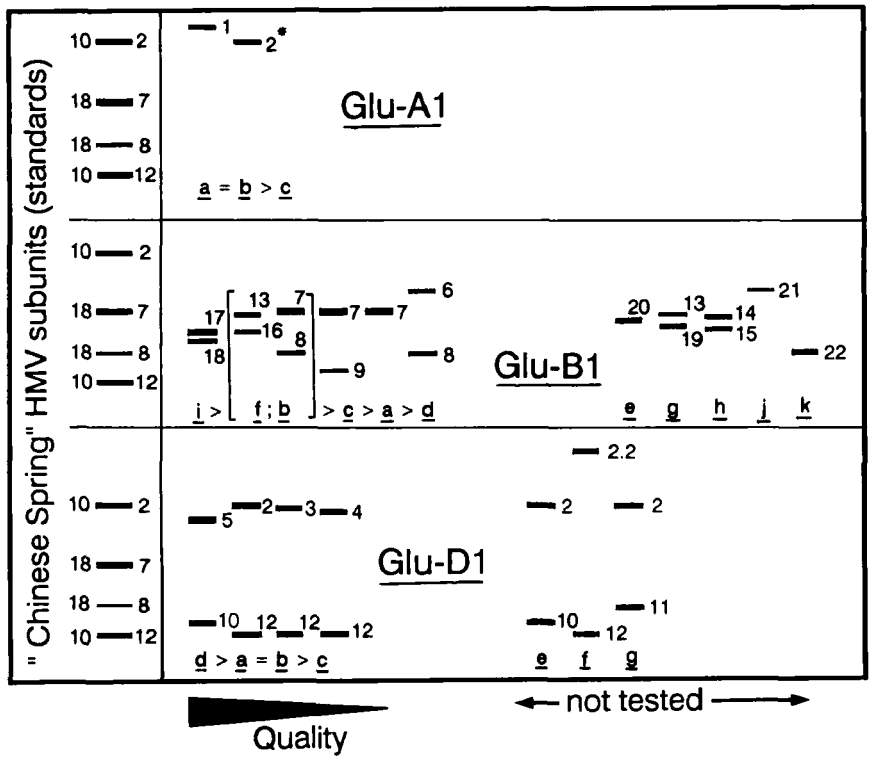


FIG. 16. Allelic variation in HMW subunits of glutenin at the three gene loci and its relation to breadmaking quality as found by Payne and co-workers. Subunits were fractionated by SDS–PAGE, with the direction of migration from top to bottom of the figure. The subunit pattern of the control Chinese Spring has been triplicated on the left to allow comparison of relative mobilities. Lowercase letters refer to the allele designations of Payne and Lawrence (1983), and ranking according to assessed quality is shown (Payne *et al.*, 1984a). It should be noted that additional alleles have been identified since this diagram was first published.

2. *LMW (B and C) Glutenin Subunits*

In contrast to the HMW subunits, the LMW glutenin subunits are encoded by genes on the short arms of chromosomes 1A, 1B, and 1D, these loci being designated *Glu-A3*, *Glu-B3*, and *Glu-D3* (see Fig. 15). Allelic variation in the LMW glutenin subunits has been studied for more than 200 cultivars by Gupta and Shepherd (1990) using two-step one-dimensional SDS–PAGE. A total of 40 different B and C subunits were detected, the number in a given cultivar ranging from 7 to 16. The subunits could be divided into 20 band patterns, which

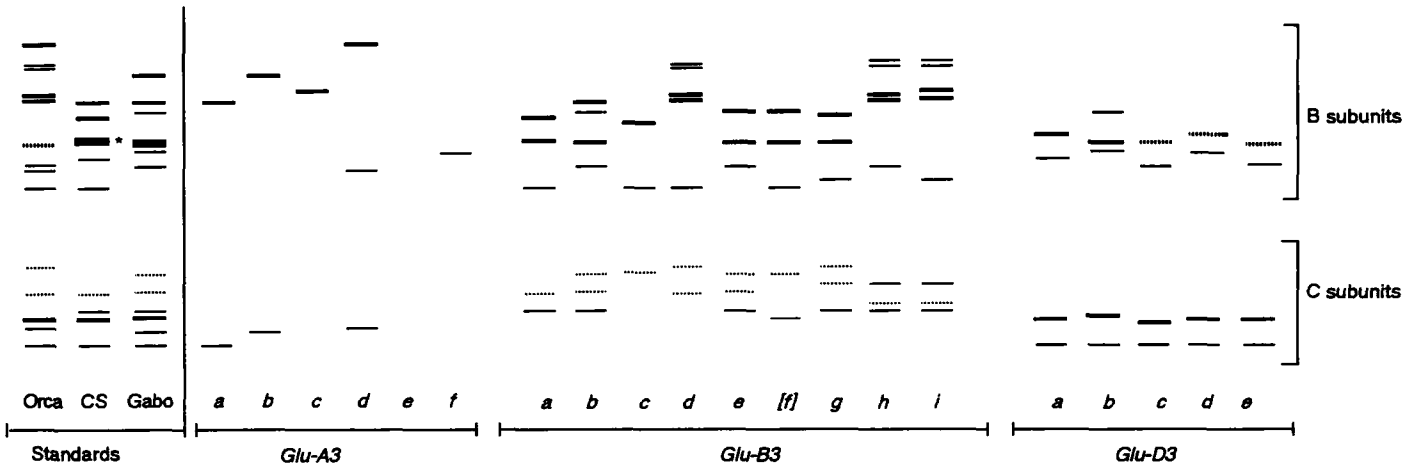


FIG. 17. The three groups (from the A, B, and D genomes, left to right) of B and C LMW glutenin subunit combinations identified by two-step SDS–PAGE analysis of over 200 bread wheat varieties, together with the patterns for three reference varieties, Orca, Chinese Spring (CS) and Gabo. Dotted lines represent faint bands. Patterns a and b in each group are from Chinese Spring and Gabo, respectively (Gupta and Shepherd, 1990).

could be classified into three groups based on their mutual exclusiveness, with 6, 9, and 5 patterns. Many allele designations and the band patterns for three standard cultivars on the extreme left are shown in Fig. 17. By analyzing substitution lines, it was determined that the different patterns in the groups were controlled by genes on chromosomes 1A, 1B, and 1D, respectively. The least number of subunits was controlled by chromosome 1A and about 40% of the cultivars examined contained no band controlled by this chromosome. The greatest polymorphism is shown by chromosome 1B. According to the scheme (Fig. 17), Chinese Spring (CS) has the designation *Glu-A3a, Glu-B3a, Glu-D3a,* as it contains "a" alleles from the loci on each of the three group 1 chromosomes. There is also evidence that some LMW subunits are controlled by genes on the group 6 chromosome (particularly 6DS) based on their solubility in ethanol and capacity to form parallel lines in diagonal electrophoresis (Gupta, 1989).

3. *Triticins*

The genetic control of triticins has been studied by Singh and Shepherd (1985) using nullisomic–tetrasomic and ditelocentric lines of the variety Chinese Spring. These proteins are encoded by genes on the short arms of chromosome 1A (*Tri-A1* locus) and 1D (*Tri-D1* locus) as shown in Fig. 15. The slowest moving band (Tri-1) of the triticins is controlled by genes on chromosome arm 1DS and the fastest moving band (Tri-3) is controlled by genes on 1AS. The band with intermediate mobility (Tri-2) is a hybrid aggregate of the subunits controlled by 1DS and 1AS.

4. *HMW Albumins*

Analysis of aneuploid stocks of the variety Chinese Spring has shown that the HMW albumins of 69, 63, 60, and 45 kDa are controlled by genes on the chromosome arms 4DL, 4AL (4BL following the current nomenclature), 5AL,

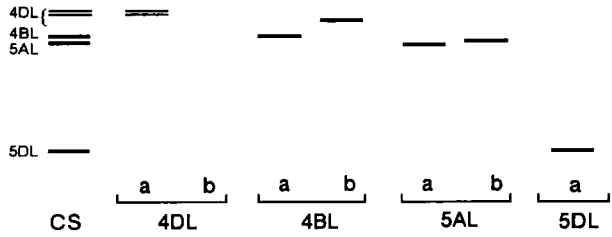


FIG. 18. The two-step electrophoretic patterns of HMW albumin bands in bread wheat. The Chinese Spring (CS) HMW albumin bands are included for reference and represent the first pattern (a) in each group (Gupta *et al.*, 1991c).

and 5DL, respectively (Gupta *et al.*, 1991c). Two-step SDS–PAGE examination of total proteins from 207 bread wheat cultivars revealed two groups of band patterns (designated "a" and "b") that were mutually exclusive. Because the patterns designated "a" corresponded to Chinese Spring, with known genetic control, the chromosomal control of the other patterns could be deduced and are illustrated in Fig. 18.

C. MONOMERIC PROTEINS

1. Gliadins

Genes coding for the gliadin proteins are located on the short arms of groups 1 and 6 chromosomes (Wrigley and Shepherd, 1973; Brown *et al.*, 1981). The group 1 chromosomes control all the ω-gliadins, most of the γ-gliadins, and a few of the β-gliadins, whereas genes on the group 6 chromosomes code for all the α-gliadins, most of the β-gliadins, and some of the γ-gliadins. The genes coding for gliadin proteins occur as a single complex locus on each of the short arms of groups 1 and 6 chromosomes rather than at two or more loci (see Fig. 15). In addition to the linkage that this signifies between gliadin genes, there is also tight linkage between gliadin genes and genes coding for the LMW glutenin subunits on group 1 chromosomes. It has been found that groups of gliadins are characterized by high stability such that they remain unchanged throughout repeated generations (Sozinov and Poperelya, 1980). These groups, within which there is no recombination, have been called "blocks" by Sozinov and co-workers (see, e.g., Metakovsky *et al.*, 1984). There is also evidence that the above picture for gliadin loci is not so straightforward and that there is at least one other locus controlling ω-gliadins, which is located approximately midway between the centromere and the *Gli-1* loci on chromosomes 1A and 1B (Galili and Feldman, 1984; Metakovsky *et al.*, 1986)

2. Albumin/Globulins

The genetic location of genes that control the many types of nongluten proteins that occur in the wheat endosperm has been ably reviewed by García-Olmedo *et al.* (1982). Genes for the major albumins and globulins of wheat have been assigned to chromosome groups 3, 4, 5, 6, and 7. Of interest is a recent report in which a family of endosperm globulins with unusual solubility properties has been located on the short arms of the group 1 chromosomes of wheat and related species (Gomez *et al.*, 1988). The interest lies in their possible use as markers in genetic manipulation of the more functionally important gluten polypeptides.

The location of genes for other wheat proteins, such as purothionins and CM proteins, has been determined, but as they are minor proteins and have not so

far been shown to be important in functionality, they are not further considered. For more information, the reader is referred to the review by García-Olmedo *et al.* (1982).

V. COMPOSITION/STRUCTURE

A. AMINO ACID COMPOSITION

Representative data for the amino acid composition of different groups of wheat proteins are summarized in Table IV. Solubility-related parameters calculated from amino acid data on different protein groups have already been discussed in Section II and a direct comparison of glutenin with gliadin composition has been made in Table III. The most obvious compositional differences are between the gluten and nongluten proteins. Gluten proteins are high in glutamic and aspartic acids (mostly in the amidated forms as glutamine and asparagine) and are low in some of the essential amino acids, particularly lysine in comparison to the nongluten proteins. Even among subgroups of the same protein family, however, there are notable differences in composition. For example, the ω-gliadins are deficient in sulfur-containing amino acids, relative to all the other gliadins.

B. STRUCTURE OF MONOMERIC PROTEINS AND POLYMERIC SUBUNITS

1. HMW Glutenin Subunits

Knowledge of the structure of gliadins and glutenin subunits has increased greatly in the past few years. This has mainly resulted from cloning of their genes and deduction of amino acid sequences from the DNA sequences of the respective genes. For example, the complete set of six HMW glutenin genes has been isolated from genomic libraries of the bread wheat cultivar, Cheyenne (Anderson *et al.*, 1988; Anderson and Greene, 1989). Model structures for the x- and y-type HMW glutenin subunits are shown in Fig. 19. In general, the HMW subunits consist of three structural domains. At the N terminus, there is a nonrepetitive sequence of some 80–100 residues containing three to five cysteine residues. There is another nonrepetitive sequence of 42 residues at the C terminus (for all subunits studied), including one cysteine. Between these two regions, there are a number of repeated sequences of some 490–700 residues. Certain anomalies have been found between the amino acid sequences derived by DNA sequencing and the sizes of subunits estimated from SDS–PAGE. For example, the Dx2 and Dx5 pair of subunits has electrophoretic mobilities opposite

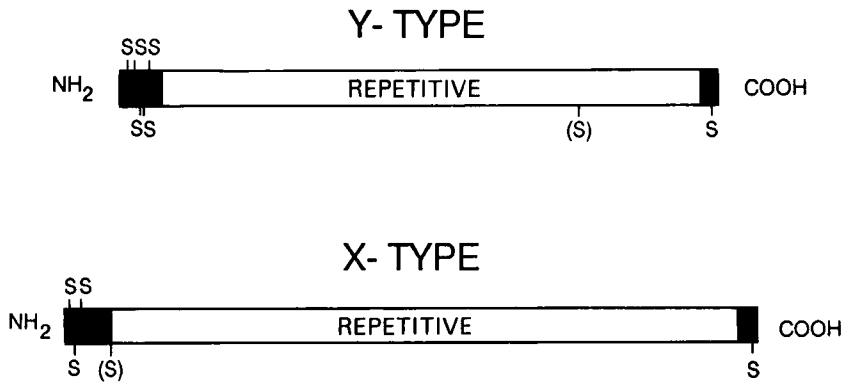


FIG. 19. Schematic illustration of the structures of the x- and y-type HMW glutenin subunits (Anderson *et al.*, 1988).

to what the number of amino acid residues from sequencing would predict. Similarly, this is the case for the Dy10 and Dy12 subunits. This unexpected behavior has been the subject of conjecture, although Goldsbrough *et al.* (1989) have produced evidence that the anomaly is related to conformational differences localized in a region near the C-terminal end of the proteins and is attributed to differences between the subunits of between one and six amino acid residues.

2. *LMW Glutenin Subunits and Gliadins*

Several DNA sequences have been reported for LMW subunits (Anderson *et al.*, 1991), including two for the cultivar Cheyenne (Okita *et al.*, 1985). This has shown that they are closely related to and, in fact, are a subfamily of the gliadins. They differ fundamentally from gliadins in their participation in intermolecular disulfide bonding with other glutenin subunits to form large polymers with a range of sizes. This difference is not fully understood, although Kasarda (1989) has proposed an imaginative scheme to explain the different polymerizing capacities of gliadins and glutenin subunits. This is illustrated in Fig. 20, where schematic representations for the structures of HMW and LMW glutenin subunits are shown together with those for γ- and α-gliadins. Because gliadins do not appear to polymerize through disulfide linkages, it is suggested that all cysteine residues participate in intramolecular bonding. This occurs because gliadins have an even number of cysteine residues. On the other hand, the glutenin subunits so far analyzed have an odd number of cysteine residues, thus providing the opportunity for intermolecular linkage. Of course, the relative positions of the cysteine residues and the manner of chain folding are also relevant.

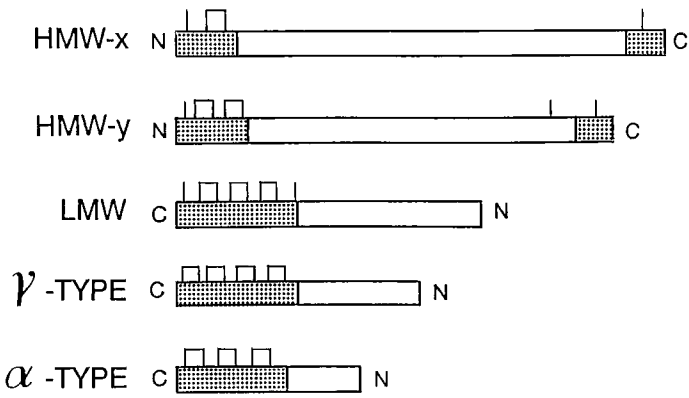


FIG. 20. Schematic representations of x- and y-type HMW glutenin subunits, LMW glutenin subunits, γ-type gliadins, and α-type gliadins as proposed by Kasarda (1989). Patterned areas represent unique sequences and plain areas represent repeating sequences. Black bars represent intermolecular disulfide linkages; linked black bars show intramolecular disulfide linkages. N, N terminus; C, C terminus. Representations are very approximately to scale.

3. *Physical Studies of Glutenin Subunits*

Shewry and co-workers (see, e.g., Tatham *et al.*, 1990) have made extensive studies of glutenin subunits and gliadins by a range of physical methods. These have included circular dichroism (CD), optical rotary dispersion, nuclear magnetic resonance (NMR), and hydrodynamic methods. CD measurements have been used to study the presence of the main structural configurations found in proteins, i.e., the α helix, β sheet, and random coil. In addition, they have emphasized the evidence for β turns, by which the polypeptide chain turns back on itself. For HMW glutenin subunits, a structure is proposed in which the central region of repeated sequences contains repeated β turns, thought to be organized into a loose spiral. It is suggested that such a structure may be intrinsically elastic and could be a key to explaining the elastic properties of doughs, contributed by the HMW glutenin subunits.

C. STRUCTURE OF POLYMERIC PROTEINS IN NATIVE STATE

Although knowledge is accumulating on the sequences and structure of the glutenin subunits, questions about how they are combined in the large polymeric molecules are yet to be resolved. It is the structure of the polymeric molecules that governs their functionality. Some ideas, referred to above, have been put forward to explain the elastic properties contributed by the HMW glutenin

subunits. However, if glutenin is broken down into its subunits (e.g., by using reducing agents in doughs), elastic properties are lost. Elasticity and high tensile strength of hydrated gluten are not properties of subunits but of the large polymeric molecules formed from them. Elasticity is a general property of all polymers above a certain size. It is associated with high molecular weight and not necessarily with any specific secondary structure. This aspect is discussed in more detail in Section VII.

1. Molecular Weight Distribution

Determinations of molecular weight and molecular weight distribution of the polymeric proteins are made difficult because of their insolubility, heterogeneity, and the inferred very high molecular weight of their largest polymers. In most size-exclusion chromatography, glutenin elutes either totally at the void volume or, if not, gives a rather monotonic curve that is difficult to transpose into a molecular weight distribution curve. Ewart (1987), in an excellent paper, has calculated a size distribution for linear glutenin molecules based on standard theory of high polymers. It is assumed that two cysteine residues, probably near the two ends of a chain (cf. Fig. 20), react with similar residues on other subunits. The subunits are treated as monomers in a polymerization reaction, leading to a calculated distribution of sizes described by the degree of polymerization (DP) distribution. The DP in turn is expressed by a single variable b, the reciprocal of the number-average DP. The distribution turns out to be identical with that produced by random degradation of an infinite molecule, b then being the fraction

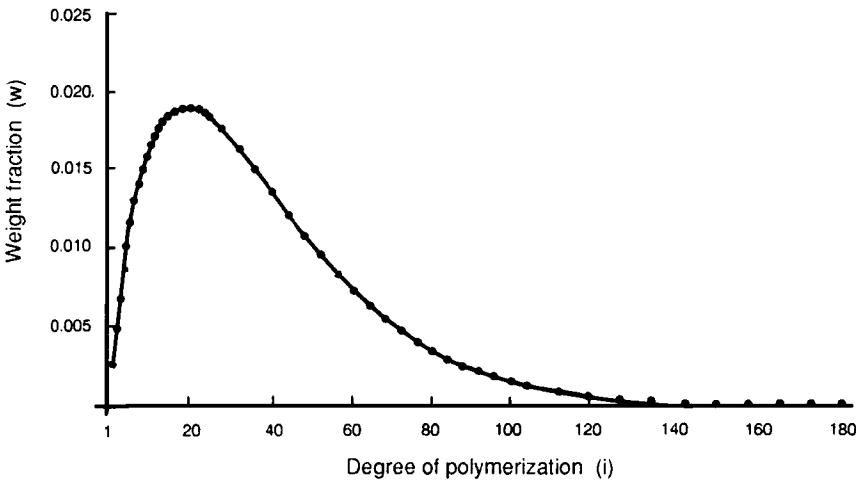


FIG. 21. Degree of polymerization distribution curve for glutenin as calculated by Ewart (1987).

of interchain disulfide bonds that are broken. The DP distribution curve calculated by Ewart for glutenin is depicted in Fig. 21. The weight fraction for DP 1, 2, and 3 is 0.014 for $b = 0.05$. Although single subunits have not been observed in native glutenin, small oligomers soluble in aqueous ethanol and extractable together with gliadins have been identified (Bietz and Wall, 1980). If an average subunit molecular weight of 50,000 is assumed, the most probable weight fraction of polymer has a molecular weight of 1×10^6 and an appreciable amount has molecular weights of several millions, consistent with estimates made from size-exclusion chromatography (Schofield *et al.*, 1983).

2. *Experimental Measurements*

The main methods that have been used to estimate molecular weights of glutenins have been exclusion chromatography, the analytical centrifuge, and viscosity. Using the ultracentrifuge, Jones *et al.* (1961) found a value of $(1.5–2) \times 10^6$ for the weight-average molecular weight of glutenin from the variety Ponca. According to the theory of Ewart (1987), this would suggest a value of 0.05 for b. The plot in Fig. 21 is based on the results of Jones *et al.* in which the weight-average degree of polymerization is 39.

Viscosity measurements are simple to perform and a useful relationship between molecular weight M (viscosity average) and intrinsic viscosity $|\eta|$ has been established (Flory, 1953).

$$|\eta| = KM^a \tag{5}$$

where K and a are constants for a particular solute–solvent system and have to be determined by calibration with compounds of similar composition whose molecular weights have been determined by an independent method. To determine intrinsic viscosity, specific viscosity (η_{sp}) is first measured, for example, by the flow time (t) of a solution through a capillary, relative to that of the pure solvent (t_0).

$$\eta_{sp} = (t - t_0)/t_0 \tag{6p}$$

η_{sp} is measured at several concentrations (c) of the polymer and η_{sp}/c is plotted against c. From this plot,

$$|\eta| = \eta_{sp}/c \quad \text{at} \quad c = 0 \tag{7}$$

Intrinsic viscosities of glutens and glutenins from 36 wheat samples have been reported by Ewart (1980). The problems associated with these measurements are well discussed by Ewart in that paper. A significant relationship was found

between intrinsic viscosity (and presumably molecular weight) of glutenin and baking quality as measured by loaf volume. Values of $|\eta|$ for glutenins varied in the range of 1.22 to 2.06 dl g^{-1}.

One way of determining molecular weight distributions of heterogeneous polymers is by separation into sharp size fractions and performing molecular weight measurements on individual fractions. This method has been applied to gluten protein fractions prepared by fractional extraction using dilute acid (MacRitchie, 1988). In order to obtain complete solubilization of protein, fractions were dissolved in NaOH solutions adjusted to pH 11.2. Although solubilization was achieved, the highly alkaline conditions appeared to cause disulfide bond scission as manifested by a decrease in viscosity with time for fractions concentrated in glutenins. It was found that the η_{sp}–time relations for fractions followed closely the equation

$$\eta_{sp} = A + B \exp(-t/b) \tag{8}$$

where A, B, and b are constants. Application of Eq. (8) enabled estimation of η_{sp} at zero time, taken as the time the solution and protein fraction were first mixed. An example of the change in η_{sp} with time is shown in Fig. 22 for fraction 6 (FN 6) from the variety Mexico 8156 at two concentrations. Characterization of this fraction has been reported elsewhere (MacRitchie, 1989). A viscosity-average molecular weight of 203,000 was calculated. The fraction was concentrated in glutenins but also contained monomeric proteins. Sharper fractions are needed for successful application of the method. Apart from measuring molecular weights, the method also provides a way of following the kinetics of reduction of the intermolecular disulfide bonds of glutenin.

3. Arrangement of Glutenin Subunits in Native Molecules

The arrangement of subunits in the glutenin polymers is conjectural. Present thinking on glutenin structure has been summarized by Kasarda (1989) and by Ewart (1990). Some relevant facts can be stated with confidence:

1. The amounts of the LMW (B and C) subunits are present in excess of the HMW (A) subunits in normal cultivars. An approximate ratio of 3:1 (LMW:HMW) is indicated, but this ratio is variable between cultivars (Gupta *et al.*, 1992).
2. As the ratio of HMW:LMW glutenin subunits increases, the molecular weight distribution curve shifts toward higher molecular weights, assuming the same DP distribution curve.

Ewart (1990) has succinctly and critically reviewed the various theories of glutenin structure. In the rather original hypothesis of Kasarda *et al.* (1976), specific secondary forces are given prominence but extensive studies of high-

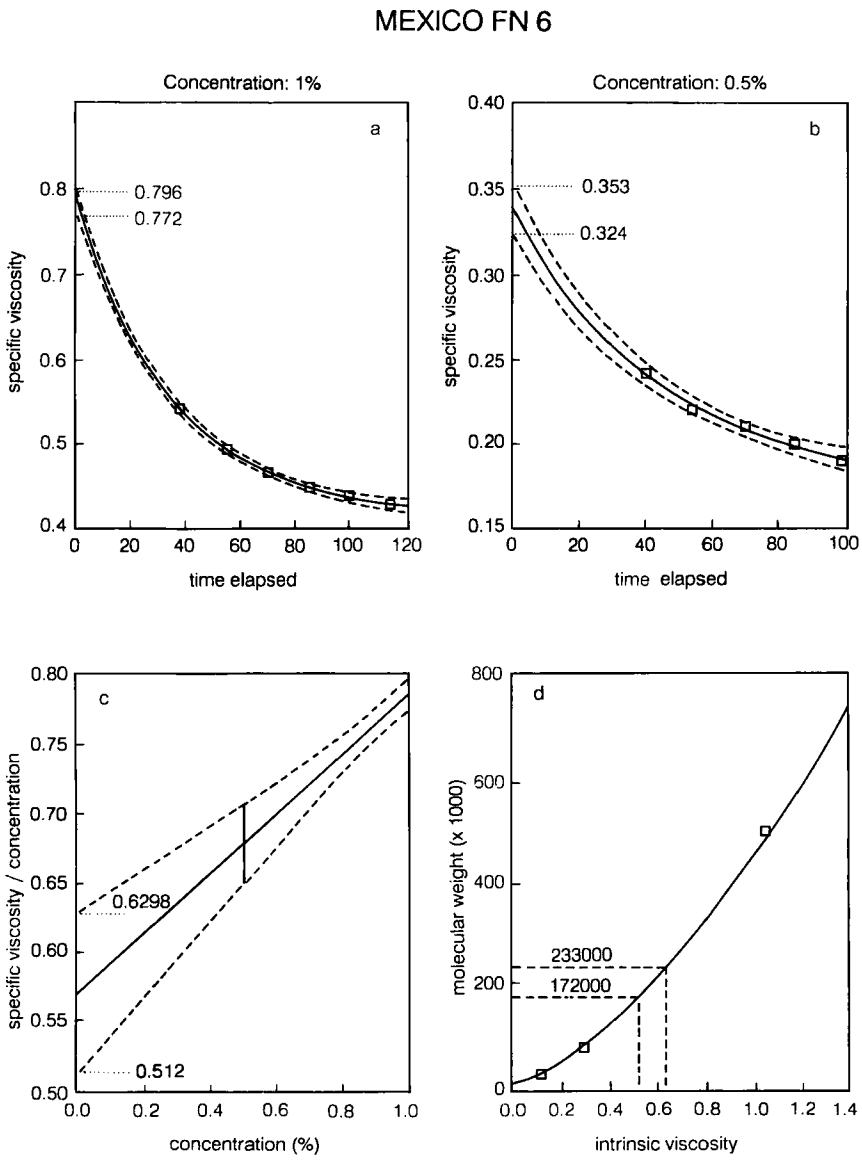


FIG. 22. Specific viscosity–time curves for solutions of gluten protein fraction 6 (FN 6) from the variety Mexico 8156 (MacRitchie *et al.*, 1991) at two concentrations, 1.0% (a) and 0.5% (b) in NaOH solution at pH 11.2. These results and others are used to evaluate the intrinsic viscosity (c) and thus the viscosity-average molecular weight of the fraction by a calibration curve using proteins of independently measured molecular weights (d) (MacRitchie, 1988).

polymer properties in recent times demonstrate that covalent molecular structure is of overriding importance. Branching was postulated by Bietz and Huebner (1980) and Bietz and Wall (1980) and a branched structure for glutenin has been proposed by Graveland *et al.* (1985). This was based on studies of fractions from size-exclusion chromatography and their analysis in terms of subunit composition, numbers of S—S and SH groups, and the effects of partial reduction. Based on this evidence, a model was proposed in which HMW glutenin subunits were linked end to end to form the backbone and strings of LMW subunits branched off from the y-type HMW subunits.

Ewart (1987, 1990) has presented evidence that the most likely structure is a linear arrangement of subunits and that subunits are linked randomly. When polymerization is branched rather than linear, the weight fraction is predicted to decrease with increasing DP and monomers would be the most abundant species, even on a weight basis (Flory, 1953). This is certainly not what is observed (cf. Figs. 7 and 8), in support of a linear rather than a branched structure. The rheological and mixing properties of doughs are also not consistent with a branched glutenin (Ewart, 1990). This is because a branched glutenin should not need development by mixing since it could not be oriented and would only need thorough wetting to reach maximum development.

The possible role of chain termination in the synthesis of glutenin polymers has been raised by Kasarda (1989), although it was stressed that this mechanism is not essential. It was suggested that some of the C subunits (M = 30,000–40,000) might act as chain terminators whereas the B subunits (M = 40,000–55,000) may be primarily chain extenders. Another question is whether some of the HMW nonprolamins polymerize with glutenin subunits. As discussed in Section III, these compounds appear mainly to associate mutually. However, it is still possible that some participate in polymerization with glutenins. If so, their manner of incorporation into the polymers and the resulting effects on functionality would be useful to know. Fractions of low solubility containing high proportions of nonprolamins in association with glutenins have been reported to have negative effects on dough properties and breadmaking (MacRitchie, 1987; Graveland *et al.*, 1982).

VI. COMPOSITION/FUNCTIONALITY RELATIONSHIPS

Wheat has a wide range of end uses and the properties required vary from one product to another. Because it would become too unwieldy to try to relate protein composition to all these products, the discussion will be restricted to dough properties and breadmaking. Nevertheless, the approaches described are generally applicable to other products.

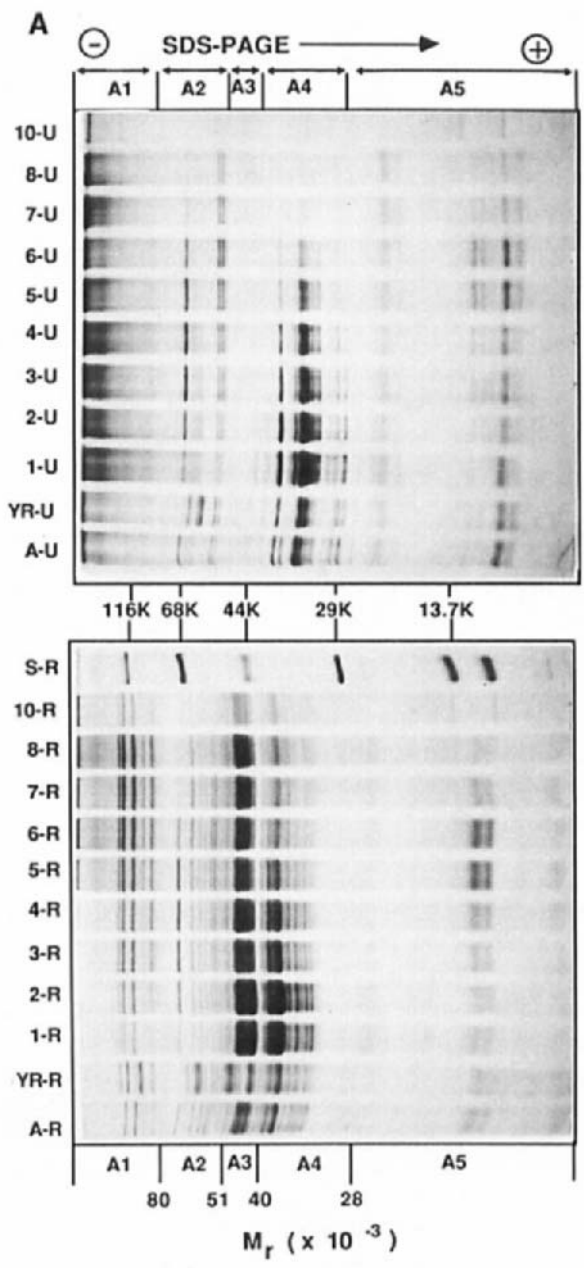
The search for a molecular basis of wheat flour functionality has involved contributions from many scientists over a long period. Several different and complementary ways of tackling the problem have been tried. Some of the conclusions that have emerged from the different approaches are discussed below.

A. RECONSTITUTION METHODS

The aim in reconstitution experiments is to separate wheat flour components and then either to (1) vary their amounts in a given flour or to (2) interchange equivalent components between flours of contrasting properties. The success of the method depends on carrying out the different steps without altering the functional properties of the components (Finney, 1943; MacRitchie, 1985). By using the first approach, Finney (1943) showed that bread loaf volume, in an optimized baking test, increased linearly as flour protein content increased from 8 to 22%. The slopes of the regression lines varied from one variety to another, indicating that differences in baking quality between varieties were due to differences in protein composition. This has been generally confirmed using the second approach of interchanging the protein (more particularly the gluten protein) between flours of differing baking quality (MacRitchie, 1978). Similar reconstitution experiments have shown the gluten protein to be responsible for the rheological and mixing properties of flour doughs (Finney, 1979; MacRitchie, 1980a). Once it has been established that it is largely responsible for differences in functionality, the protein can then be further fractionated in order to evaluate the contributions of specific fractions (using the first approach) and to pinpoint the fraction(s) that contribute(s) most to the differences (second approach).

1. Addition of Fractions

In testing the effects of addition of gluten protein fractions to a control flour, a general trend was found for fractions prepared from a number of flours by sequential extraction in dilute HCl (MacRitchie, 1987). This trend is illustrated in Fig. 23 for fractions from the variety Cook. The SDS–PAGE patterns of the fractions in reduced and unreduced conditions are shown for comparison (MacRitchie *et al.*, 1991). The SDS–PAGE patterns of reduced proteins are conveniently divided into five main groups of bands, A1–A5 (Fullington *et al.*, 1987). Bands in the A1 region correspond to HMW glutenin subunits and those in A5 correspond to monomeric albumins/globulins. In the other regions, there is some overlap between the different proteins, but region A2 contains mainly ω-gliadins, A3 contains mainly LMW glutenin subunits, and A4 contains mainly α-, β-, and γ-gliadins. The percentages of protein in each of the five regions for each fraction are shown in Table V together with corresponding data for the


A
SDS-PAGE
A1
A2
A3
A4
A5
10-U
8-U
7-U
6-U
5-U
4-U
3-U
2-U
1-U
YR-U
A-U
116K
68K
44K
29K
13.7K
S-R
10-R
8-R
7-R
6-R
5-R
4-R
3-R
2-R
1-R
YR-R
A-R
A1
A2
A3
A4
A5
80
51
40
28
M_r (x 10^{-3})

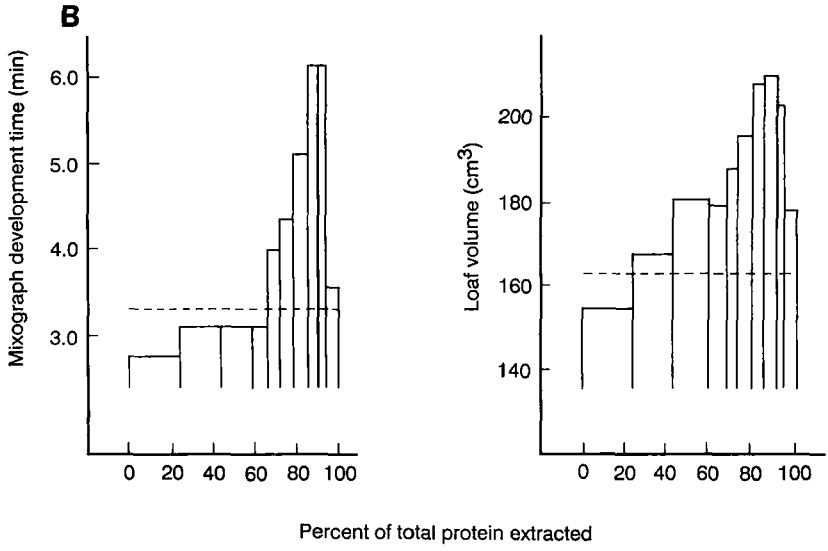


FIG. 23 Continued

effects of the fractions on two parameters, the mixograph peak development time and the loaf volume in a rapid optimized bread-baking test. The effects on functionality were evaluated by addition of sufficient amounts of each fraction to a control flour to increase the protein content by one percentage point.

The results show that the earlier fractions, which are concentrated in gliadins, reduce dough strength as measured by the mixograph peak development time. In contrast, increasing glutenin content of the sequentially extracted fractions is accompanied by increase in both dough development time and loaf volume. The trend is reversed for the latest fractions. For these latest extracted fractions, the HMW glutenin subunit content (A1) decreased slightly and the amino acid composition also has been reported to change from that expected for gluten proteins toward that more typical of nonprolamins (MacRitchie, 1987).

FIG. 23. (A) SDS–PAGE patterns of gluten protein fractions from the variety Cook, prepared by fractional extraction in dilute HCl. U, Unreduced proteins; R, reduced proteins; YR and A refer to total protein extracts from varieties Yecora Rojo and Anza, respectively, used as controls. Numbers 1–10 refer to sequence of fractions obtained by successive extractions. Chromatograms have been divided into regions A1–A5 corresponding to the five main groups of bands, and molecular weights indicated by molecular weight markers are shown (MacRitchie *et al.*, 1991). (B) Effects on mixograph development time and loaf volume in a rapid optimized bake test of addition of the fractions (whose SDS–PAGE patterns are shown in A) to a control flour so as to increase the protein level by one percentage point. In A, fractions 1–10 correspond to fractions in order of extraction (i.e., from left to right) and the percentage of the total protein in each fraction can be evaluated from the bar graph. Dashed lines show values for control flour (MacRitchie, 1987).

TABLE V

QUALITY AND COMPOSITION DATA FOR GLUTEN PROTEIN FRACTIONS[a] FROM VARIETY COOK

Fraction	Final pH of supernatant	Total protein (%)	Protein in fraction (%)	Mixograph peak development time (min)	Loaf volume (ml)	Relative areas (%)					Glutenin[b] (%)
						A1	A2	A3	A4	A5	
Control (Condor)	—	—	—	3.21	162	—	—	—	—	—	—
1	5.59	23.0	88.8	2.64	154	6.2	5.5	28.9	44.3	15.1	35.7
2	5.50	20.4	91.2	3.02	167	9.4	7.1	26.6	43.9	13.0	49.1
3	5.21	16.4	89.9	3.02	179	12.0	10.2	24.5	36.4	16.9	36.9
4	5.06	7.7	88.6	3.02	178	11.8	10.2	25.4	34.6	18.0	52.1
5	4.84	6.0	87.5	3.90	187	13.3	11.6	22.2	26.8	26.2	49.0
6	4.66	5.2	87.3	4.28	195	15.4	11.7	23.7	23.0	26.1	54.2
7	4.16	7.1	87.0	5.03	207	18.9	10.6	27.5	21.3	21.7	66.5
8	3.65	5.7	81.2	6.04	209	18.7	7.9	30.3	23.6	19.5	68.9
9	3.29	2.4	81.4	6.04	192	16.9	7.3	28.4	24.7	22.7	70.7
10	—	6.1	36.7	3.46	167	15.1	6.8	26.6	25.7	25.9	72.5
Composite of all fractions						11.6	8.3	26.6	35.6	18.0	48.8

[a]From MacRitchie *et al.* (1991)

[b]Percent glutenin was obtained from the densitometry of sodium dodecyl sulfate–polyacrylamide gel electrophoresis patterns for the reduced and unreduced fractions.

2. *Interchange Experiments*

If we have x flour samples and each is separated into n components, the number of possible distinct combinations of fractions is x^n. Thus, for two flours, each separated into three equivalent fractions, this number is 2^3, or 8. Two of the eight combinations are simply the original two flours reconstituted to their original composition. An essential condition for valid interchange experiments is that these reconstituted flours must retain exactly the functional properties of the original ones.

Interchange experiments of this type have shown that protein (especially the gluten protein) controls both the physical dough properties (e.g., mixing characteristics) and breadmaking potential of wheat flours (Finney, 1943; MacRitchie, 1978, 1985). Several studies have also reported the effects of interchanging protein fractions between flours of different quality (Hoseney *et al.*, 1969; Booth and Melvin, 1979; MacRitchie, 1980a). The results have been discussed in detail previously (MacRitchie, 1984). The interpretation of these interchange experiments is sometimes equivocal because the compositional data of the fractions are not available. This aspect has been discussed by Chakraborty and Khan (1988). Some results where three approximately equivalent fractions were interchanged between two flours of differing baking quality are summarized in Table VI. The data appear to parallel the behavior shown in Fig. 22; i.e., fraction 2 from Table VI (considered to be mainly glutenin), accounting for the main difference in quality, appears to correspond to the glutenin-enriched fractions, which show the largest effects in Fig. 23.

TABLE VI

CONTRIBUTIONS OF GLUTEN PROTEIN FRACTIONS TO DIFFERENCES IN LOAF VOLUME[a]

Fraction	Percentage of total gluten protein	Percent contribution to loaf volume difference
1. Earliest extract (concentrated in gliadin)	37	0
2. Intermediate extract (concentrated in glutenin)	40	70
3. Residue (concentrated in glutenin)	23	30

[a]Data are for two flours and are found by interchange. From MacRitchie (1980a).

B. RESULTS OF SURVEYS

1. Qualitative Relationships

Many correlation studies have been reported in which flour properties have been related to specific aspects of protein composition. Pioneering work by Payne and co-workers (see, e.g., Payne *et al.*, 1987b), established that certain HMW glutenin subunits were associated with positive effects on flour properties whereas others were associated with negative effects. One example is the allelic variation at the *D1* locus of bread wheats, where alternative pairs of subunits 5 + 10 (contributing good quality) and subunits 2 + 12 (giving poorer quality) have been contrasted. Based on analyses of a large number of varieties, a scoring system for HMW subunits has been developed (Payne, 1987) in which individual subunits are graded with numbers based on quality evaluations. A given variety can then be assigned a Glu-1 score, which is the sum of the contributions of each of the three HMW loci. The HMW score has been found to have more influence in some sets of wheats than others (MacRitchie *et al.*, 1990). It may be that when we are dealing with relatively strong wheats having large quantities of glutenin, the effects of allelic variation may be swamped by the quantitative factors discussed in Section VI,B,2. Studies of durum (tetraploid) wheats showed that superior quality, based on elastic recovery of gluten, was almost always associated with a particular gliadin electrophoretic band, denoted band 45. Similarly, poorer quality flours were invariably associated with another electrophoretic band, band 42 (Damidaux *et al.*, 1980). Recent work has tended to show that these gliadin bands are markers for quality and that the functional differences are caused by quantitative differences in LMW glutenin subunits to which these gliadins are genetically linked (Payne *et al.*, 1984b; Autran *et al.*, 1987).

A preliminary approach to ranking LMW glutenin alleles in order of quality has been reported by Gupta *et al.* (1991a) along the lines used by Payne and co-workers (1987b) for the HMW alleles. The data presented from a study of two sets of bread wheats indicated that variation in *Glu-3* alleles of glutenin in addition to *Glu-1* alleles was needed to explain more fully the differences in extensigraph parameters.

The correlation of certain proteins with quality is of potential value in plant breeding because screening of progenies can be based on simple detection of proteins by a technique such as electrophoresis or by the use of antibodies (Skerritt and MacRitchie, 1990). However, it is probably too simplistic to expect that functionality can be explained only in terms of qualitative differences in protein composition. Differences in quantities of classes of proteins must also be important. It is true that minor differences in protein composition are vital in determining biological specificity such as enzyme activity. Physical properties, by contrast, depend more on factors such as molecular weight and molecular

weight distribution. This explains why gliadin fractions from different flours behave similarly in regard to functionality. On the other hand, glutenins have a much greater capacity for variation because of their polymerizing property. Moreover, by analogy with high polymers, it is expected that variation in the relative amounts of the monomeric and polymeric proteins would be important in determining the physical properties.

2. *Quantitative Relationships*

The achievement of close to complete and consistent solubilization of wheat protein by sonication and measurement of the proportions of its three main classes by SE-HPLC (see Section II) has allowed a more accurate evaluation of the quantitative composition/functionality relationships of flours than was previously attainable. In surveys to date, examination of the correlation matrices of protein composition with functional parameters has revealed that only peak I protein from SE-HPLC is important (Singh *et al.*, 1990b; Gupta *et al.*, 1991d). Where high correlations were found with peak II and peak III proteins, this occurred because of the close relationship with flour protein content, which in turn is highly correlated with peak I protein content (Gupta *et al.*, 1991d). For the purpose of simplicity, we shall make the approximation that peak I protein is equivalent to glutenin. It was found that two measurements of composition related to flour quality parameters (Gupta *et al.*, 1992). These were the percentage of glutenin in the protein (PG) and the percentage of glutenin in the flour (FG). These can be determined from SE-HPLC profiles and the flour protein content (FP) and are defined as follows:

$$\text{PG} = (\text{Peak I area/total area of chromatogram}) \times 100$$

$$\text{FG} = (\text{FP} \times \text{PG})/100$$

The extensigraph is an instrument that has been widely used for assessing dough properties. In principle, its measurement approximates to a tensile strength test (see Section VII). A cylinder of dough is stretched at a fixed rate until it breaks, while the force of resistance is continuously monitored. Two of the main parameters measured are the maximum resistance (R_{max}) and the extensibility (Ext). These parameters are illustrated in Fig. 24.

Extensigraph data (Ext and R_{max}) plotted against PG and FG for flours from 15 hexaploid wheat varieties grown at six nitrogen fertilizer levels are shown in Fig. 25 (Gupta *et al.*, 1992). In all, 84 samples were studied, 6 being omitted from the trial because of insufficient quantity. The plots of Fig. 24 indicate that Ext and R_{max} depend differently on protein composition. Ext is highly correlated with FG (slightly better than with FP), whereas R_{max} correlates better with PG.

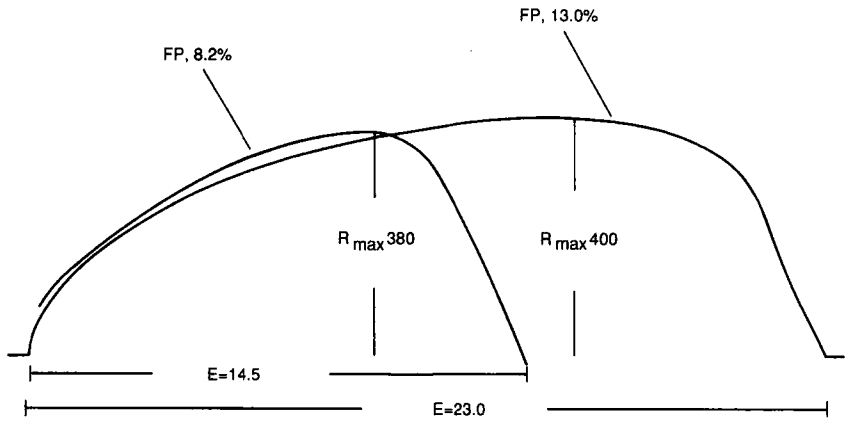


FIG. 24. Extensigrams of two flour samples from the same variety grown at two sites and having different flour protein (FP) contents showing the two main measurements, extensibility (E) and maximum resistance (R_{max}).

The correlation coefficients (r) are given in Table VII. It appears from this that Ext depends on the total amount of glutenin in the flour whereas R_{max} relates to the ratio of polymeric to monomeric proteins. It is notable that whereas some 70% of the variation in Ext is accounted for by the FG ($r^2 = 0.69$), only 44% of the variation in R_{max} can be attributed to the PG ($r^2 = 0.44$).

The clustering of points corresponding to different varieties in the R_{max} versus PG plot suggests that another varietal characteristic is contributing to the variation in R_{max}. Application of the Payne HMW glutenin score shows that three varieties that clearly have higher R_{max} values than are predicted by the line of best fit (Fig. 25) have high scores, viz., Halberd (8.5), Timgalen (8), and Mexico 8156 (10). In contrast, three varieties having R_{max} values below the line have low scores, viz., Israel M68 (5), Chile 1B (6.5), and WW15 (5). The low scores for the latter three varieties arise, at least in part, because all three are null at the *Glu-A1* locus; i.e., they do not have HMW glutenin subunits encoded by the 1A chromosome. This suggests that these varieties may be relatively deficient in HMW subunits. Confirmation has been obtained for the two most extreme varieties, Halberd and Israel M68. The glutenin composition for three samples of each of these two varieties measured by SDS–PAGE/densitometry (Gupta *et al.*, 1992) shows that the ratio of HMW:LMW glutenin subunits is appreciably higher in Halberd than in Israel M68 (Table VIII).

It thus appears that two main factors govern this measure of dough strength. Provided that the ratio of HMW:LMW glutenin subunits remains within a close range for a series of varieties being surveyed, a good correlation should be obtained between R_{max} and PG. However, if there is a wide variability in the

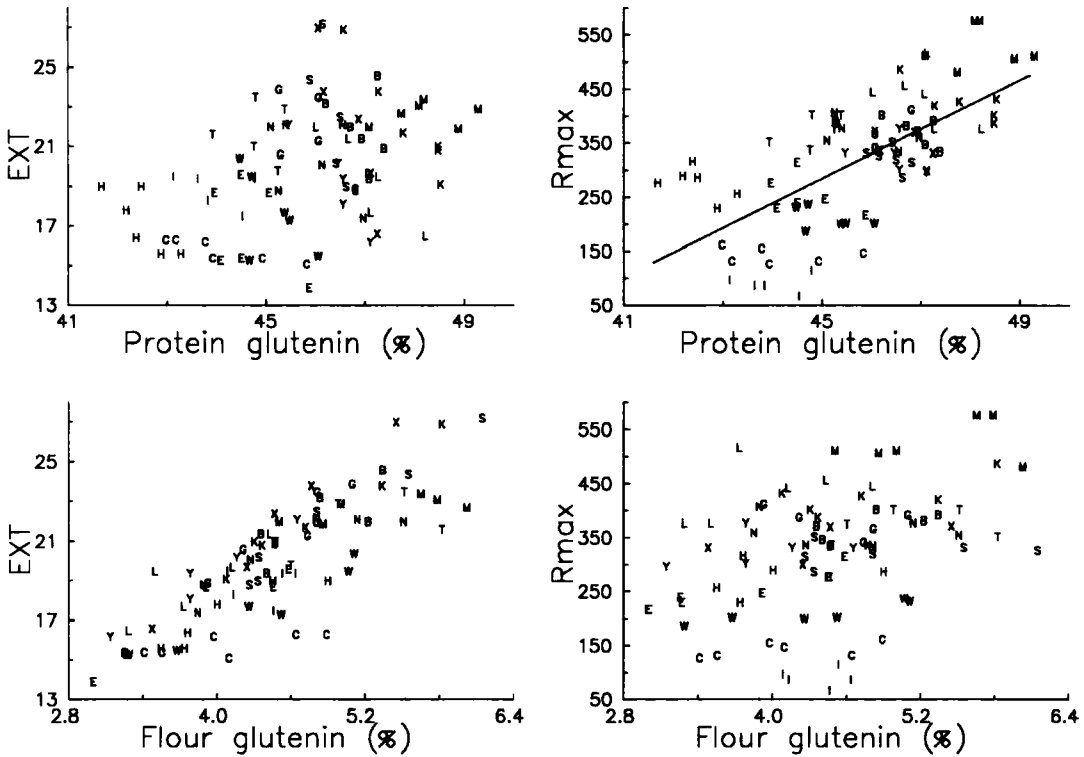


FIG. 25. Extensibility and maximum resistance as a function of protein glutenin content (top) and flour glutenin content (bottom) for 15 varieties grown at six nitrogen levels (n = 84, six samples being omitted because of insufficient quantity). Varieties are identified by symbols as follows: C (Chile 1B), N (Condor), K (Cook), E (Egret), B (Gabo), G (Gamenya), H (Halberd), I (Israel M68), M (Mexico 8156), L (Olympic), S (Osprey), X (Oxley), T (Timgalen), Y (Wyuna), and W (WW15). The line of best fit is shown for R_{max} versus protein glutenin. Correlation coefficients for linear regression are shown in Table VII (Gupta *et al.*, 1992).

TABLE VII

CORRELATION COEFFICIENTS FOR LINEAR REGRESSION OF QUALITY PARAMETERS[a]

Quality parameter[b]	Correlation coefficients (r)[c] vs. PG	vs. FG	vs. FP
Ext	0.392***	0.831***	0.744***
FDDT	0.083	0.826***	0.809***
L.V.			
(long-fermentation bake test)			
15 cv	−0.135	0.684	0.725***
14 cv[d]	−0.007	0.897***	0.877***
R_{max}	0.665***	0.392***	0.241*
MDDT	0.605***	0.127	−0.012
L.V. (rapid optimized bake test)	0.616***	0.436***	0.297**

[a]From Gupta *et al.* (1992).

[b]Ext, Extensibility; FDDT, farinograph dough development time; L.V., loaf volume; R_{max}, maximum extensigraph resistance; MDDT, mixograph dough development time.

[c]n = 84; PG, percentage of glutenin in protein; FG, percentage of glutenin in flour; FP, flour protein content.

[d]One variety, which underperformed because of excessive mixing requirements, was omitted from statistical analysis.

TABLE VIII

COMPOSITION OF PEAK I POLYMERIC PROTEIN[a]

Cultivar	HMW glutenin (%)	HMW nonprolamin (%)	LMW glutenin (%)	LMW nonprolamin (%)	HMW:LMW glutenin[b]
Halberd N-2	20.4	10.5	63.1	6.0	0.32
Halberd N-4	21.6	11.4	60.0	7.0	0.36
Halberd N-5	20.6	9.6	62.7	7.1	0.33
Israel M68 N-1	11.8	9.8	70.6	7.8	0.17
Israel M68 N-2	11.4	8.6	74.3	5.7	0.15
Israel M68 N-3	14.9	8.5	68.1	8.5	0.22

[a]Compositions determined by densitometry of SDS–PAGE patterns for two cultivars, each grown at three nitrogen levels. From Gupta *et al.* (1992).

[b]Average for the Halberd cultivars, 0.34; average for the Israel cultivars, 0.18.

ratio, a high correlation may not result. For example, if only points for Halberd and Israel M68 (Fig. 25) are considered, a negative correlation between R_{max} and PG would have been obtained because of the overriding effect of the glutenin subunit composition.

Correlation coefficients of other flour quality parameters with FG and PG are summarized in Table VII for the same 84 flour samples. Farinograph dough development time and loaf volume in a long-fermentation bake test, like extensibility, appear to relate better to FG, whereas mixograph dough development time and loaf volume in a rapid optimized bake test resemble R_{max} in their dependence on PG. Another interesting result from this survey is that, for those parameters that relate to FG, the r values are higher with FG than with FP. This suggests that the well-established relationships between parameters such as loaf volume and flour protein may be reflecting more fundamental relationships with flour glutenin.

3. *Environmental Effects*

Until now, the effects of genotype variation on functionality have been discussed. Interesting information has also been obtained by studying environmental effects. Two variables in particular have been systematically studied. These are changes producing varying levels of available nitrogen and available sulfur. Nitrogen variability affects mainly flour protein content whereas sulfur variability affects protein composition (Wrigley *et al.*, 1984).

In an optimized bake test, loaf volume increased linearly with increasing flour protein content, the slopes of the regression lines being characteristic of the variety for a large number of varieties grown over several crop years in the Great Plains areas of the United States (Finney and Barmore, 1948; Fifield *et al.*, 1950). Water absorption also increases linearly with increasing protein content and the slopes of the regression lines differ between varieties (Finney, 1979). Water absorptions varying between 55 and 62% for flours of 10% protein content and between 63 and 78% for flours of 18% protein have been reported (Finney, 1979). Oxidation requirements of flours (i.e., the amount of potassium bromate to be added for optimum performance) similarly increased appreciably with increasing protein content and were greater for short mixing than for medium-long mixing flours. Moss *et al.* (1981) measured a number of quality parameters of flours from the wheat variety Olympic that had been grown under different nitrogen and sulfur fertilizer levels. Grain sulfur concentrations varied from 0.08 to 0.18%. Flours of low sulfur content gave excessively tough doughs having high values for R_{max} and long mixograph development times. Changes in protein composition measured by SDS–PAGE of these samples have been reported by Fullington *et al.* (1987). As flour sulfur content was reduced, the sulfur-poor proteins increased relative to the more sulfur-rich proteins, as may be expected.

Thus, ω-gliadins and HMW glutenin subunits increased proportionately. The proportion of HMW glutenin subunits was strongly positively correlated with R_{max} and mixing stability. The ratio of protein in the A1 region of the gel to the protein in the A3 region (approximating the HMW:LMW glutenin ratio) was increased for the low-sulfur flours. The effects are therefore consistent with those already discussed wherein environment was constant and the genotype was varied.

C. USE OF GENETIC VARIANTS

Two main approaches for relating physical properties to protein composition have been considered. The correlative approach is based on surveys of large numbers of grain or flour samples, but caution needs to be exercised as correlations do not always reflect cause–effect relationships. The fractionation and reconstitution approach, if used correctly, is a more direct way of obtaining information. One disadvantage is that, because of the complexity of wheat protein, it is not easy to obtain pure fractions suitable for giving results capable of simple interpretation. It requires some ingenuity in the design of the experiments and needs independent checks to establish that interpretations are valid. It is not essential to have pure components. The role of pure components can be evaluated from the effects of mixtures in the same way as the weight of an unknown substance can be deduced from a series of weighings on a beam balance using combinations of calibrated weights.

Another complementary method that has become available in recent times has been the use of genetic variants from sets of near-isogenic lines in which certain proteins or subunits are either varied or not expressed. A genetic fractionation is thus utilized and the effects of varying the amounts of a given type of protein or subunit can be followed.

1. HMW Null Lines

In this more critical approach, Payne *et al.* (1987a) have used near-isogenic lines (NILs) in which glutenin subunit genes have been transferred into the genome of a common donor variety by repeated backcrossing. In this way, a series of lines was produced based on the variety Sicco, which differed only in the HMW glutenin subunit composition. Sicco has the HMW subunit composition 1 (*A1*), 7 + 9 (*B1*), and 2 + 12 (*D1*). Replacement of subunits 2 + 12 by 5 + 10 resulted in an increased bake-test loaf volume and increased mixing stability. Removal of subunit 1 (creating a null at the *A1* locus) from the NIL control caused only a small reduction in loaf volume, from 1643 to 1633 ml. Removal of subunits 5 + 10 reduced loaf volume to 1453 ml and removal of subunit 1 together with subunits 5 + 10 caused a reduction to 1328 ml.

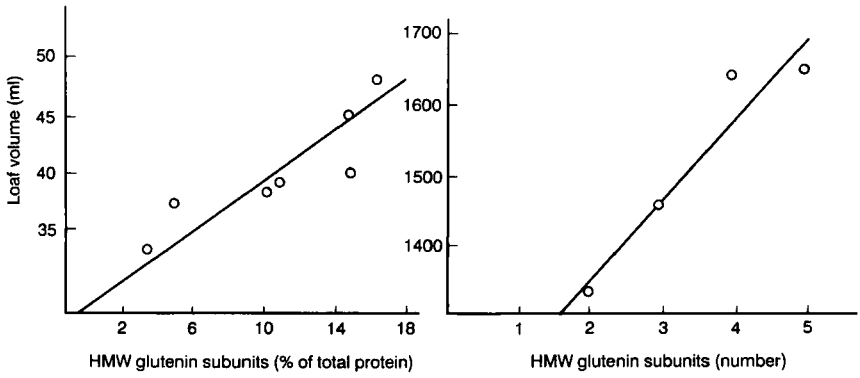


FIG. 26. Bake-test loaf volume as a function of quantity of HMW glutenin subunits in flour using near-isogenic lines to vary the quantity. Left, results of Lawrence *et al.* (1988); right, results of Payne *et al.* (1987a).

Another set of lines (HMW null lines) was derived from a mutant line of one cultivar (Olympic) null at the *Glu-B1* locus and an isogenic line of a cultivar (Gabo) null at the *Glu-A1* and *Glu-D1* loci (Lawrence *et al.*, 1988). Eight lines differing in the number and types of HMW glutenin subunits were produced. As the number of subunits in these lines was reduced from the full complement (five) to zero, mixograph development time and bake-test loaf volume fell dramatically and linearly with the proportion of HMW subunits measured by densitometry of electrophoretic gels. The effects, together with those reported by Payne *et al.* (1987a), are shown in Fig. 26 and tend to indicate that the overriding factor influencing baking potential is the number/quantity of HMW subunits present in the flour, at least for the specific subunits present in these lines. However, this conclusion needs to be further tested using similar lines with an extended range of allelic variation.

2. *Comparison of HMW and LMW Glutenin Subunits*

No equivalent set of LMW glutenin null lines is yet available, but a set of single, double, and triple rye translocation lines has been produced (Gupta and Shepherd, 1987). In these lines, one, two, or all three of the short arms of the group 1 chromosomes have been replaced by short arms of the diploid rye chromosome 1, in which no glutenin subunits are expressed. These lines therefore provide a set in which the HMW subunits are constant while the LMW subunits are reduced from their normal value (three sets) to almost zero. Secalins are also introduced by the rye short arms, but these are monomeric proteins and would be expected to behave qualitatively similarly to the gliadins that they replace. The two sets of lines (HMW null lines and rye translocation lines) have been

utilized to measure the relative contributions of HMW and LMW glutenin subunits to various functional properties (Gupta *et al.*, 1991b).

When R_{max} was plotted as a function of the PG (the percentage of glutenin in the protein), evaluated from SE-HPLC, the data for the two sets of genetic variants were clearly delineated (Fig. 27). The slope of the regression line was much steeper for variation in amount of HMW subunits than for LMW subunits. Another important characteristic is the handling properties of doughs. Modern bread processing requires that doughs pass through the system without encountering problems such as sticking to surfaces. Dough stickiness, measured by the tendency of a dough to stick to different surfaces (Dhaliwal and MacRitchie, 1990), was also applied to the two sets of genetic variants and the results are shown in Fig. 28. The relative effects of HMW and LMW glutenin subunits on extensigraph parameters (R_{max} and Ext), mixograph peak dough development time (MDDT), and dough stickiness score (DSS) inferred from the slopes of the regression lines are shown in Table IX.

It is clear that HMW glutenin subunits contribute more to dough quality parameters than LMW subunits when compared on an equal weight basis. This

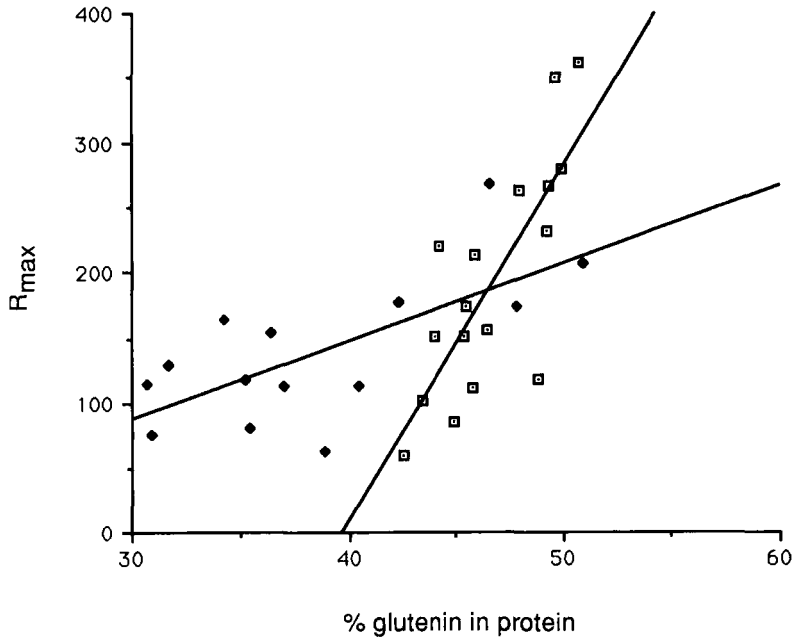


FIG. 27. Relationship between R_{max} and the percentage of glutenin in the flour protein in genetic lines varying in number/amount of HMW (shown by open symbols) or LMW (shown by closed symbols) subunits of glutenin. Lines of best fit are shown for each set of lines (Gupta *et al.*, 1991b).

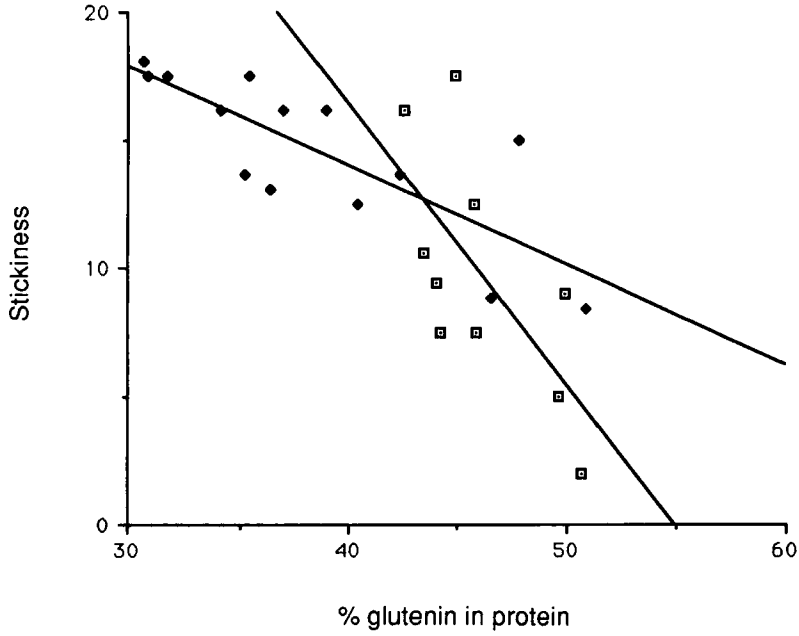


FIG. 28. Relationship between dough stickiness and the proportion of glutenin in the flour protein in genetic lines varying in number/amount of HMW (shown by open symbols) or LMW subunits (shown by closed symbols) of glutenin. Lines of best fit are shown for each set of lines (Gupta *et al.*, 1991b).

TABLE IX

RELATIVE EFFECTS OF LMW AND HMW SUBUNITS OF GLUTENIN ON DOUGH PROPERTIES[a]

Variables and methods	R_{max} (BU)	Ext (cm)	MDDT (min)	DSS
SE-HPLC				
1% change in percentage of peak I due to				
HMW subunits	27	0.5	0.6	−1.1
LMW subunits	6	0.2	0.1	−0.4
Densitometry/SDS–PAGE				
1% change in percentage of				
HMW subunits	6	0.2	0.15	−0.4
LMW subunits	2	0.1	0.03	−0.1

[a]As inferred from the slopes of the regression lines. From Gupta *et al.* (1991b).

agrees with the results discussed in Section VI,B,2. As mentioned in other parts of this review, the greater effects of HMW subunits may be related to their capacity to form larger glutenin polymers (cf. Section VI,B), but more experimental evidence is required to confirm this hypothesis.

3. Biotypes

Certain cultivars consist of two or more biotypes that differ in the protein(s) expressed at one locus. Such sets of genotypes can be regarded as near-isogenic lines, thus making them suitable for examining protein-quality relationships. Biotypes differing only in one (or one pair) of their HMW glutenin subunits or in one LMW glutenin allele are available. Establishment of pure lines of each biotype permits a comparison between the effects of the different alleles. Data on HMW glutenin subunit biotypes have been reported for a number of Australian cultivars (Lawrence *et al.*, 1987). The greatest contrast in extensigraph resistance was observed between pairs of biotypes that differed only at the *Glu-D1* locus for subunit pairs 5 + 10 and 2 + 12. From nine pairs of results, an average extensigraph resistance of 342 Brabender units (BU) for biotypes having subunits 5 + 10 was found compared to an average value of 253 BU for biotypes having subunits 2 + 12. These differences were highly significant statistically ($p = 0.001$). Other pairs of biotypes differing in subunits controlled by the *Glu-A1* and *Glu-B1* loci did not show significant differences in R_{max}, although only a few samples were tested. Some preliminary work has shown that glutenin extractability (in SDS solution without sonication) is slightly greater for flour samples having subunits 2 + 12 than for those having subunits 5 + 10 (F. MacRitchie, unpublished results). The lower extractability of the samples with 5 + 10 subunits may indicate that these subunits form larger glutenin polymers than do their 2 + 12 counterparts.

VII. MOLECULAR INTERPRETATIONS

Basically, scientists are interested in explaining physical properties of grain and flour at a molecular level, which would enable a more effective approach to manipulating these properties. Fundamental rheology is concerned with the relationships between stress, strain, and time for materials. From a rheological viewpoint, dough is complex (Bloksma and Bushuk, 1988). The viscoelastic properties are essentially those of hydrated gluten, modified by a high filler (starch) content and diluent (water). Rheological properties of a dough are highly dependent on its history, and, after preparation, it continues to undergo relaxation processes. Therefore, it is difficult to derive information from fundamental rheol-

ogy that can be applied in a practical way. However, there are standard physical dough-testing instruments that are widely used in cereal laboratories to measure flour and dough quality. Some have already been described, such as the mixograph and extensigraph. Because flour specifications are based on these instruments, it therefore appears more practical to try to analyze the measurements from these instruments in molecular terms.

A. PROCESSES IN DOUGH MIXING

1. Macroscopic Level

A more detailed description of the processes occurring during dough mixing is given elsewhere (MacRitchie, 1986). Briefly, there is an initial development stage during which the flour–water mixture becomes a coherent mass acquiring viscoelastic properties. Recording dough mixers (farinograph or mixograph) show a maximum resistance at the end of the development stage. At this point, the dough surface takes on a sheen and can be stretched into a thin sheet. Usually this corresponds to optimum mixing for breadmaking. After the peak is reached, further mixing causes a progressive decrease in the resistance, accompanied by the loss of elasticity and an increase in dough stickiness, undesirable features for processing. This stage is referred to as dough breakdown.

Water is absorbed by the flour up to a maximum (approximately 35% of the dough mass), the excess forming a separate liquid phase, containing dissolved solutes, which is dispersed throughout the dough. The protein begins as discrete coarse masses but the shear and tensile forces draw out these masses to form strands that interlink and provide the network, giving strength and elasticity. Gas cells are beaten into the dough, this process being most rapid just prior to peak dough development (Baker, 1941; Baker and Mize, 1941). These gas cells form the nuclei into which carbon dioxide diffuses during fermentation of leavened bread. Their number, size distribution, and stability determine the texture and volume of baked pan bread as well as the grain structure of other products, such as cookies. The gas cell structure in turn depends on stabilization by surface-active compounds (proteins and lipids) that are present in the dough and adsorb at the air/aqueous interface of liquid lamellae surrounding the gas cells (MacRitchie, 1976, 1980b; Gan *et al.*, 1990). The nature of the gas dispersion is very sensitive to the composition of the surface-active compounds present. For example, changes in lipid content and composition may effect large changes in loaf volume and texture of pan bread (McCormack *et al.*, 1991), internal structure of cookies (Clements, 1980), and characteristics of flat breads (Quail *et al.*, 1991). In the case of pan bread, lipids are not essential for good baking performance, showing that stabilization of gas bubbles is achieved by the proteins alone.

2. *Molecular Level*

Flours vary in the times their doughs take to reach peak development and in the steepness of the breakdown, a measure of mixing stability. This variability is governed by differences in protein composition (see Section VI). Ultimately, this variability should be understandable in terms of processes occurring at a molecular level.

a. Development Stage. The development of a dough by mixing, apart from variables such as energy input, water content, and temperature, is a function of its polymeric protein; differences from one wheat variety to another reflect variation in the glutenin component (Finney, 1979; Huebner and Wall, 1976). At a macroscopic level, a continuous network is created that provides the viscoelastic properties. At a molecular level, what appears to be involved is the extension of glutenin molecules by the shear and tensile stresses exerted by the mixer. Because glutenin has a spectrum of molecular weights, a progressive extension of the molecules probably occurs, beginning with the smallest polymers and ending with the largest. A synthetic flour made from gliadin and starch shows practically no dough development properties.

An important parameter is the average maximum extension ratio (λ_{max}) defined as the ratio of the length of the fully extended, freely molecular-jointed chain (r_{max}) to that of the unperturbed molecule (r_0).

$$\lambda_{max} = r_{max}/r_0 \tag{9}$$

λ_{max} can be shown to increase with the square root of the degree of polymerization, i.e., essentially with the square root of the molecular weight (Smith *et al.*, 1984)

The mixing action extends the molecules against the frictional forces acting in the dough; at the same time, a retractive mechanism begins to operate as a result of the entropic restoring forces tending to return the molecules to their energetically favored (unperturbed) configurations. The time required to mix a dough to peak development (at a given mixing intensity) is thus expected to be related to the size of the largest glutenin molecules present. In fact, a mixograph curve may prove to be a sensitive measure of the molecular weight distribution of the glutenin in a flour. The other variable affecting mixing properties is the mixing intensity. Work by Kilborn and Tipples (1972) showed that if either mixing speed or work input level is below certain critical values, the resulting dough performs unsatisfactorily in breadmaking. As the dough strength of a flour increases, the critical values become higher.

It seems probable that a steady state may be reached during mixing at or below the critical mixing intensity, where the rate of extension of glutenin molecules

by the mixer acting against the frictional forces becomes equal to the rate of retraction due to the restoring entropic forces. If, for a given mixing speed, the degree of extension is insufficient to give optimum dough properties for a strong flour, then mixing intensity would need to be increased. The phenomenon of "unmixing" (Tipples and Kilborn, 1975) may also be rationalized by similar considerations. Unmixing occurs when a dough is mixed to peak consistency at high speed and then mixed for a further time at a speed well below that required for optimum development. The dough then deteriorates, assuming properties similar to a dough that has been undermixed. Because of the lower mixing intensity, the forces causing extension of molecules are outweighed by the retractive forces. The mixing action facilitates the diffusional processes required for the extended molecules to revert, at least partially, toward their unperturbed configurations. The effect is illustrated in Fig. 29. A similar effect occurs when a dough is rested, but the diffusional processes (involving molecular segments) are not so rapid in the viscous medium as in a dough that is being mixed, and the configurations are to a certain extent frozen.

The different dependencies of mixograph and farinograph peak development time on flour composition (Section VI) may relate to the different mixing actions of the two instruments. In the farinograph, the dough is being continuously sheared by the blades. Possibly this action depends more on dough extensibility and this would explain the dependence of farinograph development time on the amount of glutenin in the flour (FG). The pin-mixing action of the mixograph, on the other hand, corresponds more to tensile stresses encountered by the pins as they strike the dough. It may therefore relate more to the resistance of the dough to stretching and, like this parameter, correlates with the polymeric/monomeric protein balance (PG) and with the polymeric protein composition.

b. Breakdown. At its optimum state, a dough is coherent and the protein strands have formed a continuous network. Its strength depends on entanglement

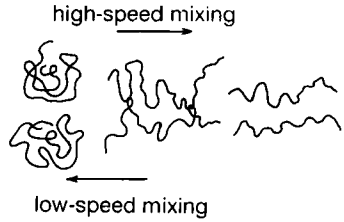


FIG. 29. Schematic representation of changes in configuration of glutenin molecules in response to mixing and "unmixing" (MacRitchie, 1986).

points between the long chains acting as transient cross-links (for a discussion, see MacRitchie, 1980b). If mixing is continued past the peak development time, the dough undergoes weakening. Two effects can be identified. The first is reversible. As molecules become orientated in the direction of shear, entanglements loosen, thus reducing the points of support when the dough is subjected to stress. A second effect, which is irreversible, involves the scission of covalent (disulfide) bonds causing the largest molecules to be broken down (see discussion in Section II,F).

c. Disulfide–Sulfhydryl Interchange Reactions. The role of disulfide–sulfhydryl interchange reactions remains a controversial topic. Thiol-containing peptides of molecular weight in the order of 2000 and smaller molecules such as glutathione occur in flour (Sullivan, 1940; Jones and Carnegie, 1969) and are potentially capable of participating in interchange reactions. When used at low levels (ppm) in doughs, disulfide bond reducing agents (e.g., cysteine and dithiothreitol), sulfhydryl oxidizing agents (e.g., iodate and bromate), and sulfhydryl blocking agents (e.g., *N*-ethylmaleimide, or NEMI) produce large effects on the rheological properties. This has led to speculation about their mechanisms but many experimental observations have not been satisfactorily explained with regard to the role of sulfur-containing amino acid residues in the proteins of dough (for discussions, see Bloksma and Bushuk, 1988; Pomeranz, 1988). All the above-mentioned reagents increase the extractability of flour protein (usually measured in dilute acetic acid solution) during dough mixing (Mecham, 1968; Tsen, 1969; Tanaka and Bushuk, 1973a). With reducing agents, the effect on extractability was observed using flour–water suspensions and did not require dough mixing (Tsen, 1969). However, both oxidizing agents and sulfhydryl blocking agents required mixing to effect appreciable increases in extractability, and these were above those caused by dough mixing in their absence. The scission of the largest glutenin molecules responsible for the effect of dough mixing on extractability has been discussed in Section II,F. This scission can be achieved by reducing agents, which accounts for their ability to increase extractability from flour–water suspensions. The role of sulfhydryl oxidizing and blocking agents is not so clear. If the scission of disulfide bonds as dough is mixed is partially reversible, then a reagent such as NEMI could conceivably act by blocking the reverse reaction, thus accelerating the extractability. Disulfide content decreased when a dough was mixed (Tanaka and Bushuk, 1973b) and was marginally lower in the presence of NEMI and iodate. The magnitudes of the decreases appear consistent with expectation, remembering that large proportions of the disulfide bonds in flour protein are intramolecular and that only the largest glutenin polymers are split at centrally placed interchain bonds (see Section II,F).

B. EXTENSIGRAPH PARAMETERS

Several load-deformation instruments are in standard use in cereal laboratories. The two most common are the extensigraph and the alveograph. The extensigraph (see Fig. 24) simulates closely a tensile stress measurement. Tensile stress curves are shown in Fig. 30 for materials of different characteristics. The main reason why the extensigraph trace differs from the traces shown in Fig. 30 is that no allowance has been made for the change in cross-sectional area of the dough piece as it is stretched, so that effectively the force and not the stress (force/unit area) is being recorded. Two main extensigraph measurements have been considered in Section VI, R_{max} and Ext. R_{max} corresponds approximately to the ultimate strength of tensile stress measurements and Ext approximates to the elongation at break.

1. Studies of High Polymers

Fundamental studies of stress–strain relationships for polymers should be of direct relevance to the interpretation of dough properties. Because of the industrial importance of polymers, considerable research has been carried out in this area. In spite of this, the relationships between physical properties and molecular structure are still not well understood, though much progress has been made (Nunes *et al.*, 1982). It has generally been established that the properties of uncross-linked amorphous polymers are largely determined by the molecular weight (MW) and the molecular weight distribution (MWD), assuming that other variables, such as temperature and composition, are constant. Furthermore, it has become widely recognized that, since the initial concepts introduced by Bueche (1965), molecular entanglements are the basis of the material properties of these types of polymers (Bersted, 1979; Turner, 1982).

It has been found that mechanical properties can be correlated with the number-average molecular weight, M_n, according to the relation proposed by Flory (1945):

$$\text{Property} = A + B/M_n \tag{10}$$

where A and B are constants. A relationship of this type has been found to describe the tensile strength (σ) of polymers quite well, provided the MWD is narrow, i.e.,

$$\sigma = \sigma_\infty(1 - M_T/M) \tag{11}$$

σ_∞ is the limiting tensile strength for high molecular weights, M is the molecular weight of the sample, and M_T is a threshold molecular weight. Below a molecular

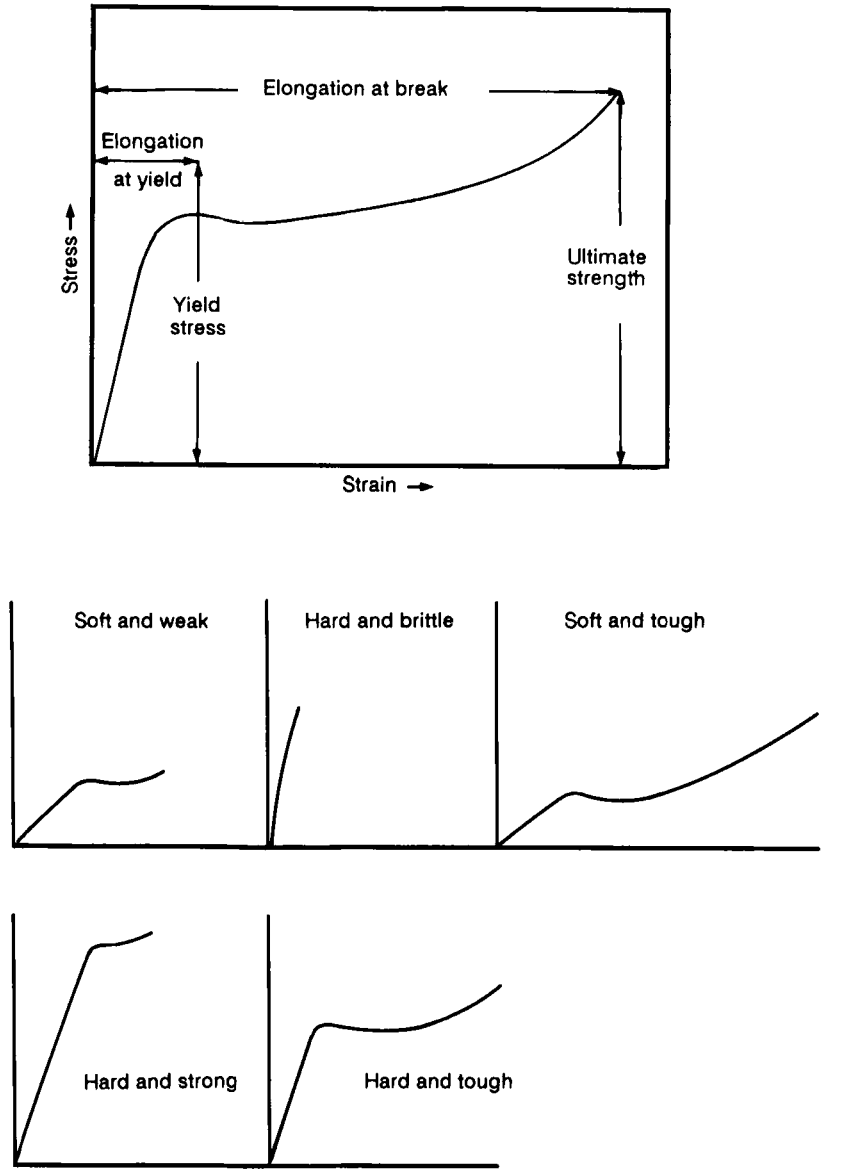


FIG. 30. Generalized tensile stress–strain curves for polymeric materials showing main parameters that are measured (top diagram) and some typical curves for polymers with a range of properties (Billmeyer, 1971).

weight of M_T, molecules do not participate in effective entanglements and therefore do not contribute to tensile strength.

However, in the case of polydisperse polymers, traditional measures of molecular weight (such as number average and weight average) have not been able to account for tensile properties (Bersted and Anderson, 1990). A new parameter, termed the failure property parameter, has been proposed by Bersted and Anderson (1990) to describe the tensile strength of polydispersed polymers. This parameter is based on the assumption that it is the entanglement network in an amorphous polymer that contributes to the strength properties. The following equation, a modified form of Eq. (11), was proposed:

$$\sigma = \sigma_\infty(1 - M_T/\overline{M}^*_n)\phi \tag{12}$$

where the expression $\phi(1 - M_T/\overline{M}^*_n)$ is termed the failure property parameter. $\overline{M}^*_n$ is the number-average molecular weight for that part of the MWD greater than M_T, and ϕ is the fraction of molecular species with molecular weights above M_T. The basis of this equation is that molecules with molecular weights below M_T are ineffective in forming strength-enhancing entanglements, and this fraction of molecules behaves as a diluent of fraction $1 - \phi$. Essentially, Eq. (12) is the usual equation for the molecular weight dependence [Eq. (11)] modified by this premise.

2. *Application to Wheat Proteins*

Dough strength as measured by R_{max} appears to agree well with what is predicted by Eq. (12). The value of M_T for wheat proteins in dough has not been determined, but evidence suggests that the monomeric proteins do not contribute to dough strength (MacRitchie, 1987). The proteins in this fraction (gliadins, albumins, and globulins) probably all behave similarly and act essentially as diluents. Based on observations (Fig. 25), it was suggested that two factors appeared to govern R_{max}. One was the proportion of polymeric protein (or the PG) that may correspond approximately to ϕ in Eq. (12). The second factor is another varietal characteristic that is envisaged as being related to differences in $\overline{M}^*_n$. As the size distribution of glutenin molecules is increased, $\overline{M}^*_n$ increases and consequently the term $1 - M_T/\overline{M}^*_n$ of Eq. (12) increases. For a given value of PG, two factors could conceivably contribute to variations in $\overline{M}^*_n$. One is the HMW:LMW glutenin subunit ratio. As this ratio is increased, higher values of $\overline{M}^*_n$ would result even on the simple assumption that the DP remains the same. The data (Fig. 25 and Table VIII) indicate that higher values for the HMW:LMW glutenin subunit ratio appear to be related to higher values of R_{max}. A similar conclusion is indicated by the results summarized in Fig. 27.

The other factor is composition as it relates to the specific glutenin subunits. It is possible that certain subunits (e.g., HMW subunits 5 + 10) could contribute to higher values of $\overline{M}_n^*$ than others (subunits 2 + 12). In that case, a higher R_{max} would result even if the PG [roughly equivalent to ϕ in Eq. (12)] were the same for the two samples being compared (see discussion of biotypes, Section VI).

A schematic illustration of the application of Eq. (12) to describe dough strength (R_{max}) is shown in Fig. 31. Two parameters are highlighted. One is ϕ, which is the fraction of the protein with molecular weights above M_T. This is shown as the shaded area in the diagram. The exact value of M_T is not known. It may correspond fairly closely to the cutoff point between peaks I and II of the SE-HPLC profile as shown in Fig. 7, i.e., it coincides with PG. However, it may be that some of the oligomers eluting toward the end of peak I are below the size needed to form effective entanglements (i.e., below M_T), and this possibility is taken into account in Fig. 31.

3. *Extensibility*

The compositional and structural factors determining Ext (Fig. 25 and Table VII) are not the same as those determining R_{max}. It is important to distinguish between the extensibility, as measured for a viscoelastic material and the plas-

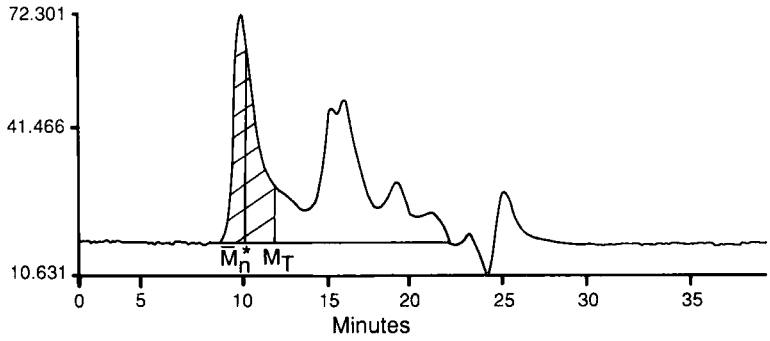


FIG. 31. Schematic illustration of application of Eq. (12) to measurements of R_{max} for wheat flour doughs based on the SE-HPLC measurement of the molecular weight distribution. M_T is the molecular weight threshold below which species are ineffective in forming strength-enhancing entanglements. The shaded portion corresponds to ϕ, the fraction of proteins having molecular weights above M_T. $\overline{M}^*_n$ represents the number-average molecular weight for the part of the molecular weight distribution greater than M_T. It should be noted that the SE-HPLC profile shown is not a true molecular weight distribution curve, since the polymeric proteins mainly elute at the void volume. However, it serves to illustrate the principle schematically.

ticity, which refers to the drawing out of a nonelastic material. Extensibility corresponds to elongation at break in a tensile stress test. For dough, it has been shown to be related to the PG (Table VII), but correlations with FG are usually much higher, accounting for some 70% of the variance. The extensibility of molecules as measured by the extension ratio [Eq. (9)] is highly relevant and large molecules are needed in view of the relationship between λ and the square root of the molecular weight. This is not the only factor, however. As a dough is stretched, protein network strands become thinner until a point is reached when they can no longer support the applied stress, and failure occurs. The total amount of protein (and particularly the effective glutenin) that forms the network strands is thus of prime importance.

After accounting for much of the variability in extensibility in terms of the flour glutenin content, some 30% of the variability still needs to be explained for the sets of flours that have been examined. Unlike the situation with R_{max}, it appears that, although high extensibility depends on the presence of large polymeric molecules, very high molecular weights may be detrimental. For example, flours in which the HMW:LMW glutenin subunit ratio is very high, as occurs as a result of sulfur deficiency (see Section VI,B), have reduced extensibility. Similarly, replacement of HMW subunits 2 + 12 by 5 + 10 decreases extensibility. Evidence has been obtained that these effects are related to shifts in the molecular weight distribution of glutenin to higher molecular weights (R. B. Gupta and F. MacRitchie, unpublished observations). It therefore appears that there is a limiting molecular weight for glutenins above which extensibility begins to be diminished. This may be rationalized as follows. When a dough piece is stretched, the transient cross-links due to entanglements provide the strength, but these are able to pull free in response to the tensile force, thus conferring the mobility required for elongation. However, when the molecular weight is increased to a certain value, the density of the entanglement network reaches a point where the transient cross-links no longer are able to slip free so easily at the imposed strain rate, causing a loss of mobility that manifests as a reduced extensibility.

One thing that needs to be remembered is that extensibility as measured by the extensigraph is not an absolute measurement. Just as tensile properties of a polymeric material, such as elongation to break, depend on the method of preparation of the sample, extensibility of a dough is influenced by its state of development. In standard methods for measuring extensibility (e.g., AACC method 54-10), the dough is mixed for a fixed time to a fixed consistency. Since different flours have different mixing requirements for optimum development, this can mean that their doughs are not being compared at the same state of development. If a flour has very long dough-mixing requirements, it may give a relatively low extensibility, because the optimum protein network has not had sufficient time to form.

VIII. BREEDING STRATEGIES

Until now, we have discussed the relationships between wheat protein composition/structure and functional properties and have summarized briefly the chromosomal location of genes coding for the major protein groups. This information now allows a discussion of how strategies can be devised for manipulating the genes, and therefore the protein composition, in order to produce grain and flour having appropriate end-use properties. This is the overall scheme depicted in Fig. 1. To illustrate the approach, several examples of problems encountered in the wheat industry will be considered and their possible solutions analyzed.

A. INCORPORATION OF ALIEN GENES

Wheat breeding is a continuous process. New varieties are being developed in order to replace those that become susceptible to disease or to meet the requirements for new or altered end-use properties. One effect of selective breeding is that it tends to lead to a restricted gene pool being available in breeding programs (Porceddu *et al.*, 1988). As a result, there is interest in introducing genes from alien species so as to exploit possible benefits such as better adaptability, increased disease resistance, improved yield, or increased protein content. Examples of the introduction of disease resistance from alien species into hexaploid wheats have been well described by García-Olmedo and co-workers (see, for example, Doussinault *et al.*, 1983; Delibes *et al.*, 1987).

The introduction of genes that contribute beneficial characteristics also involves a risk that they may be accompanied by other genes that have detrimental effects. The rye translocation lines are examples. These are lines in which one of the short arms of a group 1 chromosome of wheat has been substituted by the short arm of chromosome 1 of the diploid rye (1R). Most useful of these are 1B/1R lines derived from hexaploid wheats; i.e., the short arm of chromosome 1B has been replaced. These lines confer increased resistance to stem, leaf, and stripe rust (Mettin *et al* 1973) as well as offering increased yield potential. However, breeding programs to exploit 1B/1R translocation lines have been hindered because of an inherent quality problem associated with reduced dough strength and excessive dough stickiness (Zeller *et al.*, 1982).

Increased flour water absorption and a high content of soluble pentosan have been suggested as possible causes of sticky doughs in 1B/1R lines (Zeller *et al.*, 1982). However, no definite correlations of pentosans with stickiness have been found (Dhaliwal *et al.*, 1988; Martin and Stewart, 1986). Using fractionation and reconstitution techniques and comparing translocation lines with their recurrent parents, MacRitchie *et al.* (1986) and Dhaliwal and MacRitchie (1990) showed that most of the dough stickiness (about two-thirds) originated in dif-

ferences in the gluten protein and somewhat less (one-third to one-half) in the water-soluble flour fraction. Two factors were suggested as contributing to the problem. First, loss of the LMW glutenin subunits encoded by the 1B chromosome short arm results in a reduced proportion of glutenin in the protein. This is illustrated in the SE-HPLC profiles of Fig. 32A, where the proportion of glutenin (peak 1) is reduced from 34.8% in the recurrent parent cultivar (Cook) to 25.9% in its 1B/1R derivative (Sun 89D).

The SE-HPLC profiles for soluble (Fig. 32B) and insoluble (Fig. 32C) fractions of gluten protein from the same lines, prepared by differential solubility in HCl, are shown. Interchange of the soluble fractions showed that the gluten soluble fraction of the 1B/1R line contributed to increased dough stickiness, whereas the gluten insoluble fractions of both flours were similar. These effects are consistent with the SE-HPLC profiles for the two sets of fractions. The profiles

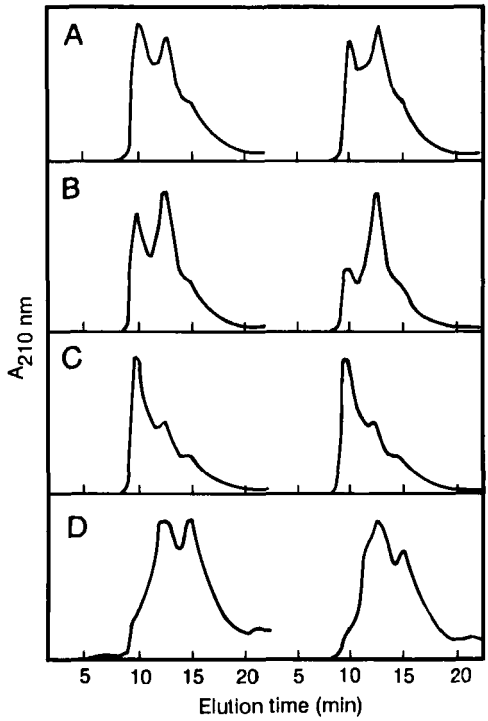


FIG. 32. SE-HPLC profiles of protein from recurrent parent variety, Cook, and its fractions (left) and its rye translocation derivative, Sun 89D, and its fractions (right). (A) Extract from whole flours; (B) gluten soluble fractions; (C) gluten insoluble fractions; (D) water-soluble fractions (Dhaliwal and MacRitchie, 1990).

are similar for the insoluble fractions, but the gluten soluble fraction of the 1B/1R line has a much lower proportion of glutenin. The second and more minor factor apparently contributing to dough stickiness is the gain of rye monomeric proteins, the secalins, by the 1B/1R line. This shows up in an increased quantity of water-soluble protein and an increased contribution to stickiness by the water-soluble fraction of the 1B/1R line (Dhaliwal and MacRitchie, 1990).

In summary, dough weakness and the accompanying poor handling properties appear to be caused by a shift in the balance of polymeric/monomeric proteins resulting from the loss of the 1B-encoded LMW glutenin subunits and the gain of the monomeric 1R secalins. This hypothesis was supported by direct experiments in which protein fractions either deficient or fortified in glutenin were added to a control flour, causing increased and decreased dough stickiness, respectively (Dhaliwal and MacRitchie, 1990).

Strategies for counteracting the deleterious effects therefore need to focus on compensating for these changes. The obvious approach is to use a recurrent parent that has exceptionally strong dough properties (e.g., a high R_{max}). Then the translocation line might be expected to have a lower yet acceptable dough strength. However, the problem is not so simple. If the parent line has a high dough strength that is largely caused by a high proportion of HMW glutenin subunits or by a favorable combination of specific HMW subunits, than it may have unacceptably long mixing requirements. Loss of the LMW subunits may not reduce the mixing requirements very much because this will shift the HMW:LMW ratio to higher values. As we have seen (Section VI), properties such as R_{max} and mixograph dough development time (MDDT) appear to depend on the proportion of glutenin in the protein (PG) and the HMW:LMW ratio. The apparent nexus between R_{max} and mixing requirements is discussed later in this section.

There are some options available for countering the problem of sticky doughs in rye translocation lines (specifically 1B/1R lines):

1. Increase the expression of HMW glutenin subunits at the *A1*, *B1*, and *D1* loci.
2. Choose alleles known to be associated with strong dough properties (e.g., HMW subunits 5 + 10 at the *D1* locus).
3. Increase the expression of LMW glutenin subunits at the *A3* and *D3* loci.
4. Select lines that have low expression for monomeric proteins, e.g., lines with relatively small numbers of gliadins or lines that are null for gliadin blocks.
5. Use rye parent lines having relatively low expression of secalins.
6. Produce wheat–rye recombinant lines that separate the dough quality defects from the desirable agronomic characters introduced by the rye.

Options 1 and 2 are attractive from one point of view. Increase of dough strength and reduction of dough stickiness are achieved more efficiently by

increasing the amount of HMW glutenin subunits (cf. Figs. 26 and 27) and probably by selection for specific HMW subunits. One approach has been to increase the number of expressed HMW subunits in hexaploids from the normally maximum value of 5 to 6 by transferring genes from the *A*-genome diploids *T. thaoudar* (P. I., Payne, personal communication) or *Triticum umbellulatum* (Faridi, 1988), in which both the x- and y-HMW subunits are expressed. This certainly should increase dough strength, but, as mentioned above, the altered balance in the glutenin subunit composition may lead to unacceptably long dough-mixing requirements. Option 3 aims to restore this balance, but there is a limit to how much compensation can be achieved, as the *Glu-B3* locus is usually responsible for the highest expression of LMW subunits of the three loci. Selection for low expression of gliadins (option 4) has the advantage that it should ensure a high proportion of glutenin without affecting its subunit balance. This is also the case in option 5, where the aim is to introduce lines having secalin variants with reduced amounts. The last option (6) is being pursued by Shepherd and co-workers (1991) using 1D/1R lines. The ideal recombinant may possibly be one in which the disease resistance genes from rye and the *Glu-D3* locus occur together on the 1D chromosome short arm, but the genes coding for secalins are absent. Linkage mapping has shown that a very small segment of 1RS chromatin is required to maintain resistance to all three rusts, and that it should be possible but difficult to separate the rust resistance genes from the secalin genes (Singh *et al.*, 1990).

B. EXCESSIVE DOUGH-MIXING REQUIREMENTS

In many breeding programs, high dough strength (as measured by R_{max}) has been targeted as a desirable quality characteristic. This is particularly the case for markets where wheat is used as a component of bread flour grists and where breadmaking methods require a high degree of inherent strength. An effect has been that this increased strength is almost always associated with considerably increased dough-mixing requirements. This can cause disruptions in processing schedules in bakeries.

Although dough strength and dough development time appear to have similar dependencies on protein composition, this nexus is not necessarily unbreakable, as is illustrated in Fig. 33. Plots of MDDT versus R_{max} are shown for data from the two sets of genetic lines described in Section VI, i.e., one set varying in amounts of HMW glutenin subunits, the other in amounts of LMW subunits. Two features are notable. First, mixing times increase with increase of R_{max}. Second, the rate of increase is appreciably higher for variation of HMW subunits. This suggests that to achieve doughs with moderate mixing times requires boosting the ratio of LMW:HMW glutenin subunits. Because this modification will tend to lower R_{max}, a concurrent compensating strategy for increasing

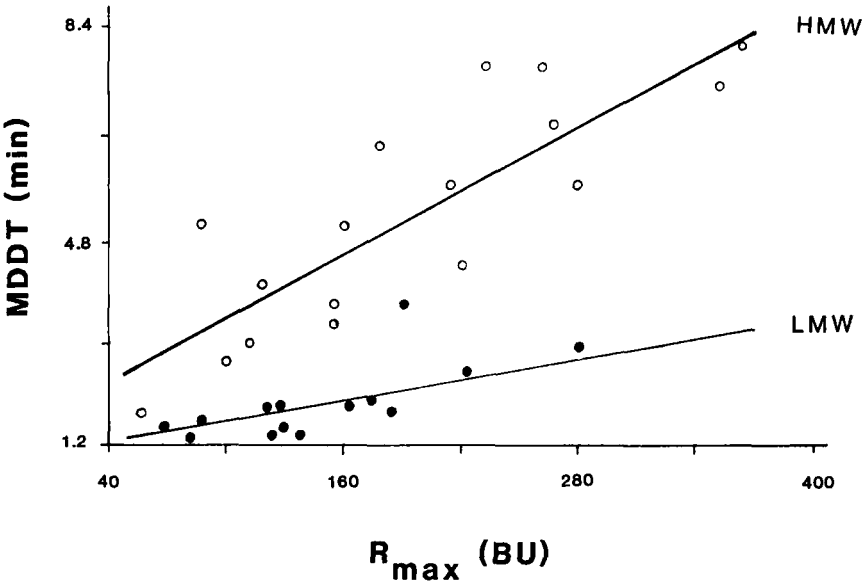


FIG. 33. Mixograph dough development time (MDDT) versus maximum extensigraph resistance (R_{max}) for two sets of genetic lines varying in the proportion of HMW (top line, open symbols) and LMW (bottom line, closed symbols) glutenin subunits. Linear regression gave the following equations: MDDT = 1.54 + 0.0195R_{max} (HMW subunit variation) and MDDT = 0.71 + 0.0087R_{max} (LMW subunit variation).

the proportion of glutenin in the protein (PG) needs to be implemented if dough strength is to be maintained. In relation to this problem, the biochemical basis for variation in relative quantities of polymeric and monomeric proteins has been discussed by Singh *et al.* (1990).

C. LACK OF EXTENSIBILITY

In addition to strength (measured by R_{max}), the capacity of a developed dough to be stretched without breaking (i.e., the extensibility) is another desirable property for breadmaking. We have seen (Section VI) that most of the variation in extensibility (about 70% for surveys carried out to date) appears to be accounted for by differences in the total amount of glutenin in the flour (FG). The protein balance in terms of the polymeric:monomeric protein ratio, the HMW:LMW glutenin subunit ratio, and the specific HMW subunits appear to be less critical (but not completely unimportant) factors than they are for determining R_{max}. This is illustrated in Fig. 23. The extensigraphs for two samples of flour of the same variety but with different flour protein contents are shown. Values of R_{max} are not very different, consistent with this parameter being determined largely

by genotypic variables (PG, HMW:LMW glutenin subunit ratio). By contrast, the extensibility of the two samples is very different due to its dependence on flour glutenin content. Thus, to some extent, extensibility is controlled by environmental effects that cause variation in protein content. A neat demonstration of this has been reported in a study by Gupta *et al.* (1989a) in which extensigraph data for families from crosses between two bread wheats were compared when the wheats were grown at two locations and thus had two flour protein levels.

However, some obvious strategies are available for enhancing extensibility in breeding programs. One is to select for high expression of glutenin (high PG), a tactic already alluded to in Section VIII,B. Simultaneously, it is important to select for glutenin composition to ensure that the molecular weight distribution is not shifted to the high molecular weights that reduce extensibility (see Section VII,B). This can be done by maintaining the LMW:HMW glutenin subunit ratio sufficiently high and by utilizing specific subunits that do not contribute to excessive dough strength (e.g., HMW subunits 2 + 12 rather than 5 + 10). The other main avenue for increasing extensibility by enhancing flour glutenin content is to select for inherently high protein content. This topic is further discussed below.

D. INCREASING PROTEIN CONTENT

Wheat crops in recent times have been characterized by declining protein levels. The reason is partly that breeding objectives have concentrated on grain yield. Increased yield usually equates to decreased protein level. Various promising lines for conferring higher protein content are being investigated. These include derivatives of the high-protein diploid *Triticum dicoccoides* and the 2B/2R translocation line (Gupta *et al.*, 1989b). When such lines are used in breeding programs, screening of progeny is needed to check whether any negative traits, such as yield penalty and poor dough properties, are introduced. This requires the techniques described previously for analyzing protein composition, such as the various HPLC and electrophoretic methods. Increase of flour protein content is usually accompanied by an increased proportion of gliadin and a decreased proportion of albumin/globulin proteins (Gupta *et al.*, 1992). This change in protein balance has nutritional implications, particularly because of the decrease in proportion of the important essential amino acid lysine.

E. CHROMOSOMAL AND GENETIC ENGINEERING TECHNIQUES

Manipulations of chromosomes, chromosome arms, and portions of chromosome arms are well-established techniques and have already been mentioned in connection with HMW nulls, rye translocation lines, and their recombinants. For use in further fundamental studies on composition/structure/functionality

relationships of wheat proteins, there is unlimited scope for developing other interesting lines. Other sets of HMW null lines and equivalent sets of LMW null lines would be useful to compare more extensively the effects of allelic variation. By crossing these lines, it should be possible to produce lines in which glutenin proteins are completely deleted. Lines that are mutant for gliadin and/or glutenin protein are valuable for clarifying the relationships between composition and quality (Lafiandra *et al.*, 1991; Novoselskaya *et al.*, 1991).

Although genetic engineering techniques have been used to introduce genes into certain plant species, including rice, no successful transformation system has been devised for wheat. Workers in this area are confident, however, that this will be achieved in the future. Work in the molecular biology area involving cloning of genes and restriction fragment-length polymorphism (RFLP) mapping is laying the foundations for genetic engineering to be applied to wheat. The breeding strategies described in this section rely on connections between specific proteins (identified by electrophoresis) and the genes that control their synthesis. Many genes control other functions that do not lead to expressed protein, such as genes for disease resistance. This is where RFLP markers have an advantage, as they can be used directly to identify the genes. It is in such areas that genetic engineering methods could have their initial greatest impact.

IX. FUTURE DIRECTIONS

Based on discussions in relation to the integrated picture depicted in Fig. 1, it is apparent that a number of useful future research areas will be developed.

A. NEW RESEARCH MATERIALS

Chromosomal engineering opens the possibility to produce novel lines valuable for research purposes. The use of HMW glutenin null lines, rye translocation lines, and biotypes have been considered. There is further potential for developing similar lines, e.g., other sets of isogenic HMW null lines based on alleles different from those already studied. This allows a more effective comparison between the effects of specific glutenin subunits. By crossing the triple HMW null line with the triple rye translocation line, tetranulls, pentanulls, and hexanulls for glutenin subunits can be produced. This work has been initiated by Dr. R. B. Gupta at this laboratory. The hexanull line (null at all three *Glu-1* and all three *Glu-3* loci) would thus be practically devoid of glutenin proteins. Similarly, lines in which gliadin proteins are systematically deleted are also possible. Bread wheat mutant genotypes in which the entire cluster of gliadin components, controlled by genes on chromosome 6A (*Gli-A2* locus), is absent have been studied by D'Ovidio *et al.* (1991), and other mutant lines for gliadins on group 6

chromosomes have been identified by Dr. E. V. Metakovsky (personal communication). These lines could be valuable for crossing with lines having high expression of LMW glutenin subunits, with the view to producing varieties with high expression of glutenins but with a high ratio of LMW:HMW subunits. As discussed in Section VIII, this could be a viable option for correcting excessive dough-mixing requirements and reduced extensibility in breeding programs. Deletion of the gliadins from the group 1 chromosome is complicated by the tight linkage between these gliadins and the LMW glutenin subunits. This linkage between gliadins and LMW glutenin subunits is an area where an increasing focus will almost certainly be directed in future investigations. Another area highly relevant to application of strategies to manipulate functional properties pertains to the question of how the deletion of different specific groups of polypeptides affects the synthesis of others.

B. STRUCTURE OF GLUTENIN

As has been discussed in this article, properties of wheat flours are largely governed by the native glutenin structure. Determination of the way different subunits are linked and resultant size distributions of glutenins should be a priority area of research. Although amino acid sequences have been inferred from DNA sequences for glutenin subunits, this approach does not ascertain the positions of interchain disulfide bonds. Classical protein chemistry is needed for this. However, because of the apparent heterogeneity of glutenin, this is not so simple as the problem of identifying the positions of disulfide bonds in pure proteins. Sequence data also give no information about molecular size distributions of the glutenin polymers. The genetic variants described above are valuable for elucidating the role of specific subunits in the formation of glutenin polymers. Partial reduction of glutenin followed by studies of the reduced proteins is also a promising approach to understanding glutenin structure and is in progress in several laboratories (D. D. Kasarda, personal communication; Ng and Bushuk, 1991). The molecular weight for effective entanglements in dough proteins is yet to be determined. This could be achieved by addition of glutenin fractions of narrow molecular weight ranges and measurement of the threshold molecular weight required to contribute dough strength to a control flour. Measurement of the number-average molecular weights of glutenins from different wheat varieties is required to test theories of dough strength, such as the theory discussed in Section VII,B. Of the colligative properties required for this determination, osmotic pressure appears to be the most viable for giving measurable values for these high-molecular-weight proteins.

The synthesis of glutenin from its subunits is a process that deserves closer attention. In postulating molecular weight distributions, it has usually been assumed that polymerization is random (Ewart, 1987; Kasarda, 1989). Although

this is a reasonable first assumption, there is some evidence that this may not be the case. This comes from the higher inferred molecular weights when specific subunits (e.g., HMW subunits 5 + 10) are present (R. B. Gupta and F. MacRitchie, unpublished results) and from the products of partial reduction, the preliminary results of which tend to indicate a nonrandom incorporation of subunits.

C. COMPOSITION/FUNCTIONALITY

More work needs to be done in relating protein composition to different functionality parameters. Three main approaches have been discussed in this article—fractionation and reconstitution, surveys in which either genotype or environment is varied, and the use of genetic variants of near-isogenic lines. Each of these approaches should be pursued, as they give essential complementary information that is needed for tackling this complex problem. There is a need for greater use of the fractionation and reconstitution approach in order to give fundamental information on the relationships between the proportions of the main groups of proteins and functional parameters (e.g., dough mixing time, dough strength and extensibility, and loaf volume potential in breadmaking). A simple way to acquire this information is to separate the flour protein from a given variety into two or more fractions. These fractions are then reconstituted in the original flour (at constant flour protein level) in differing proportions, quality parameters are measured, and protein composition is monitored by HPLC and SDS–PAGE. This approach has the advantage that it does not change either the nonprotein components or the specific proteins, as in surveys of different genotypes; it simply varies the ratios of the main protein groups. This advantage of constancy of specific proteins but alteration of their proportions can also be achieved without the need for fractionation by utilizing environmental variation to alter the balance of proteins in a given genotype. An example of this is the variation in sulfur availability described in Section VI,B. Although fundamental relationships between quality parameters and quantitative aspects of composition are established by these methods, the effects of different genetic backgrounds are required to provide information on a broader scale about the effects of allelic variability. This information is obtained from surveys of varieties with a range of properties and from studies on near-isogenic lines that vary in the expression of one or a few selected proteins or subunits.

There is a need to develop theoretical models that predict functional properties at a fundamental molecular level, such as that based on Eq. (12) for predicting extensigraph resistance. Knowledge of the dependence of different functional parameters on the molecular weight of glutenin would be of great value in tackling a problem such as breaking the nexus between dough strength and dough development time. The work of Keller and co-workers (see, for example, Keller and Odell, 1985) on the mechanism and the forces required to extend polymer

molecules in solution is highly relevant to an understanding of dough mixing and extensibility. It would be valuable to extend this work to concentrated dispersions more related to dough systems.

D. BREEDING STRATEGIES

The final test of theories of composition/structure/functionality relationships is to be able to manipulate functional properties of grain or flour in predictable ways. The implementation of strategies to test the theories as they are developed in conventional or novel breeding programs is likely to be an exciting area of cereal research in the immediate future.

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ADVANCES IN FOOD AND NUTRITION RESEARCH, VOL. 36

PLANT FOOD PROTEIN ENGINEERING

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I. INTRODUCTION

In 1991, the United Nations Population Fund forecasted that the population of the world will be 6.4 billion at the end of this century. The land available on the Earth for cultivation is limited, and it is said that a green arable zone that covers an area twice as big as Belgium turns to desert every year. Therefore, it is clear that the food harvested on the Earth will not be sufficient to feed all of the future population. Furthermore, it may be difficult to produce food stably because of the effects of global warming and abnormal weather patterns. At present, many people in developing countries are starving while many others, in the developed countries, eat excessively and develop health problems related to obesity, for example, hypertension, diabetes, artherosclerosis, and heart disease (Gurr, 1984; Yamashita and Hayashi, 1988; Watanabe, 1990; Ogihara and Mikami, 1991). The current situation demands adequate and stable food production and distribution of food that is acceptable and useful for the maintainance and promotion of human health. Animal foods are generally delicious, but ingestion of excessive animal fat causes some of the diseases mentioned above (Gurr, 1984; Dyerberg, 1986; Watanabe, 1990). It is also known that there is a relationship between the food we eat and the promotion of some types of cancers (Carroll and Khor, 1975; Carroll *et al.*, 1986). Food scientists are required to help solve these complicated problems related to foods.

Among the three main nutrients in foods, proteins will be the first to be in shortage. The high-protein foods we consume are derived from both animal and plant sources. Food proteins should be safe, economical, and nutritional, have acceptable sensory qualities, and possess functional properties and perhaps physiological activity. Nutritional properties of food proteins are determined by their content of essential amino acids. Functional properties of food proteins are properties such as dough formation, gelation, emulsification, and foaming, which also affect the sensory function of the foods. Functional properties determine the use of the proteins in foods and the acceptability of the foods (Kinsella *et al.*, 1985). Physiological activities of food proteins have recently attracted the attention of many researchers interested in the physiologically active peptides that are derived from the enzymatic breakdown of food proteins. These physiologically active peptides include opioid peptides from milk and wheat proteins (Brantl *et al.*, 1979; Zioudrou *et al.*, 1979; Yoshikawa *et al.*, 1984), opioid antagonists from milk proteins (Yoshikawa *et al.*, 1986), epidermal growth factor from milk proteins (Petrides *et al.*, 1985), cholesterol-lowering peptide from soybean proteins (Sugano *et al.*, 1990), and insulin-modulating peptide from soybean proteins (Minami *et al.*, 1990). However, it is not yet clear whether such physiologically active peptides originating from food proteins function in the human body. Therefore, physiological activity may not be important in assessing the value of food proteins.

Plant proteins are superior to animal proteins from the practical points of cost and abundance, but are usually inferior in terms of nutritional value and organoleptic considerations. However, plant proteins have a prominent place as functional food materials and can help solve the problem of excessive intake of animal fats, since plants have little fat. Moreover, plant proteins account for about 30% of the dietary protein supply in Europe and the United States and ~80–90% in Asian and African countries. In Japan, plant proteins account for about 47% of the protein supply, of which 54 and 19% come from cereals and legumes, respectively. Thus, plants are already a very important source of food proteins, and, as such, improvements in nutritional value and in functional properties of plant proteins attract scientific attention as a potential means of relieving the problems of starvation and nutritional diseases. To attain these goals, biotechnology can play an important role.

Biotechnology involves techniques such as genetic engineering, protein engineering, cell technology, and enzyme engineering. These can contribute to solving some of the problems described above. Protein engineering is a technique to modify protein primary sequences by gene manipulation. The primary sequences of a target protein can be systematically modified, thus protein engineering has the potential to become a method for enhancing the nutritional value and functional properties of food proteins. To attain this, it is necessary to meet the following goals:

1. Elucidate the physical bases and mechanisms underlying the functional properties of the target proteins.
2. Elucidate the relationships between the structure and the functional properties or digestabilities of the target proteins.
3. Characterize the structures of the target proteins.
4. Isolate the genes or the cDNAs encoding the target proteins.
5. Design the gene modifications required to improve the nutritional value and functional properties of the target proteins based on the relationships between the structure and the functional properties or digestabilities of the target proteins.
6. Prepare modified genes or cDNAs encoding the modified proteins.
7. Express the modified genes or cDNAs in microorganisms and obtain the modified proteins.
8. Evaluate whether the modified proteins have the capacity to form the correct conformation and exhibit expected properties.
9. Generate transgenic plants that produce the modified protein.
10. Feed back to (5) above.

The final aim of plant food protein engineering is to breed protein-producing crops that have high food qualities. In this article, the subject of plant food protein engineering, focusing on cereal and legume seed storage proteins, is reviewed.

II. CHARACTERISTICS OF SEED STORAGE PROTEINS

Seed proteins are classified according to their solubilities as water-soluble albumin, salt-soluble globulin, alcohol-soluble prolamin, and acid- or alkaline-soluble glutelin (Osborne, 1924). Seed storage proteins can be characterized as proteins that (1) are stored abundantly in protein bodies during seed development, (2) do not exhibit any enzymatic activities, and (3) are a source of nitrogen and carbon during seed germination. Seed storage proteins include mainly globulins in legumes and prolamins and glutelins in cereals.

A. NUTRITIONAL PROPERTIES

The protein contents of cereal seeds range from ~7 to 15%; the protein contents of legume seeds range from ~20 to ~40% (Table 1). The amino acid compositions of various cereal and legume seed proteins and the suggested pattern of amino acid requirements are listed in Table II. The data show that the amino acid compositions of seed proteins are adequate for adult requirements but do not satisfy infant and child requirements. Most cereals are deficient in lysine, threonine, and tryptophan, whereas most legumes are deficient in the sulfur-

TABLE I

COMPOSITION OF SOME CEREALS AND LEGUMES[a,b]

Food source	Protein (%)	Fat (%)	Carbohydrate[c] (%)
Cereals			
Rice	7.4	3.0	72.8
Maize	8.6	5.0	70.6
Wheat	10.5	3.0	71.4
Barley	10.6	2.8	70.8
Oat	13.0	6.2	65.3
Rye	12.7	2.7	70.4
Legumes			
Soybean	35.3	19.0	28.2
Pea	21.7	2.3	60.4
Field bean	26.0	2.0	55.9
Peanut	25.4	47.4	18.8
Kidney bean	19.9	2.2	57.8

[a]Data from Resources Council, Science and Technology Agency, Japan (1982), with permission.
[b]Dry seed basis.
[c]Fiber included.

containing amino acids, threonine and tryptophan. The protein digestability of cereal seeds is generally 75–90%, whereas that of raw and cooked legume seeds is 15–80% and 50–90%, respectively (Eggum and Beames, 1983). Therefore, the nutritional quality of seed proteins is not as high as that of egg white or meats. It is desirable to fortify seed-derived proteins, especially cereals and legumes, with lysine and sulfur-containing amino acids, respectively, or to consume a blend of these proteins.

B. STORAGE PROTEINS OF CEREALS

1. Classification

Prolamins account for about 40–50% and glutelins for about 35–40% of seed proteins in wheat (*Triticum*), barley (*Hordeum vulgare*), and rye (*Secale cereale*) (Pernollet and Mossé, 1983). The prolamins from wheat, barley, and rye are called gliadins, hordeins, and secalins, respectively, and glutelins from wheat are called glutenins. Prolamins and glutelins were initially thought to be different from each other. Glutelin consists of polymers of very high molecular weights stabilized by interchain disulfide bonds (Shewry *et al.*, 1986; Shewry and Tatham, 1990). However, when reduced, most of the constituent subunits of glutelin are alcohol soluble (Shewry *et al.*, 1986; Shewry and Tatham, 1990). Moreover, the constituent subunits of glutelin and prolamin share similar properties of

TABLE II

SUGGESTED PATTERNS OF AMINO ACID REQUIREMENTS AND AMINO ACID COMPOSITIONS OF SOME SEED STORAGE PROTEINS

	Suggested pattern of requirement[a]				Cereal[c]				Legume[d]				
Amino acid	Infant mean (range)[b]	Preschool child (2–5 years)	School-age child (10–12 years)	Adult	Rice	Maize	Wheat	Barley	Soybean	Pea	Field bean	Peanut	French bean
His	26 (18–36)	19	19	16	21	27	21	20	30	26	26	27	30
Ile	46 (45–53)	28	28	13	40	34	34	35	51	41	41	40	45
Leu	93 (83–107)	66	44	19	77	127	69	67	82	70	71	74	78
Lys	66 (53–76)	58	44	16	34	25	23	32	68	71	63	39	65
Met + Cys	42 (29–60)	25	22	17	49	41	36	37	33	24	21	32	26
Phe + Tyr	72 (68–118)	63	22	19	94	85	77	79	95	76	69	100	83
Thr	43 (40–45)	34	28	9	34	32	28	29	41	36	33	29	40
Trp	17 (16–17)	11	9	5	11	6	10	11	14	9	8	11	11
Val	55 (44–77)	35	25	13	54	45	38	46	52	47	46	48	52

[a]Data from FAO/WHO/UNU (1985), with permission. Values are in mg/g crude protein.

[b]Amino acid composition of human milk.

[c]Data from Eggum and Beames (1983), with permission of the authors and publisher. Values are in mg/g crude protein.

[d]Data from Resources Council, Science and Technology Agency, Japan (1982), with permission. Values are in mg/g crude protein.

primary structure, contents of glutamine and proline, and gene structure. Therefore, glutelin should be considered as equivalent to prolamin (Shewry *et al.*, 1986; Shewry and Tatham, 1990). According to this classification, prolamins of wheat, barley, and rye are classified into three groups, sulfur-rich prolamins, sulfur-poor prolamins, and high-molecular-weight (HMW) prolamins (Shewry and Tatham, 1990) (Table III). Sulfur-rich prolamins are the major component (~80%) and HMW prolamins account for about 10% of prolamin fractions (Shewry and Tatham, 1990).

In maize (*Zea mays*), sorghum (*Sorghum bicolor*), and millet (*Pennisetum americanum*), prolamins account for about 50–60% of seed proteins and glutelins for about 35–40% (Pernollet and Mossé, 1983). The prolamins of maize, called zeins, consist of 27-, 22-, 19-, 16-, 14-, and 10-kDa components when separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Thompson and Larkins, 1989; Shewry and Tatham, 1990). These components are classified as α-zeins (22 and 19 kDa), β-zein (14 kDa), γ-zeins (27 and 16 kDa), and δ-zein (10 kDa) based on their solubilities and primary structures (Esen, 1987; Thompson and Larkins, 1989). The α-zeins (70% of total zeins) are extracted by aqueous alcohol alone and exhibit charge heterogeneity (Thompson and Larkins, 1989). The β-zein (~5%), γ-zeins (~20%), and δ-zein (<5%) are extractable in the presence of a reducing agent and exhibit little charge heterogeneity (Thompson and Larkins, 1989). The 27-kDa zein has also been called glutelin-2 (Prat *et al.*, 1985) because of its solubility in water containing a reducing agent. The β- and γ-zeins are rich in sulfur-containing amino acids.

Rice (*Oriza sativa*) and oats (*Avena sativa*) differ from the cereals described above in that their major storage proteins are not prolamins. The major storage proteins of rice are glutelins (~80%) and those of oats are globulins (50–80%), whereas the prolamins in rice and oats account for only ~8 and 10–20%,

TABLE III

CLASSIFICATION OF PROLAMINS OF WHEAT, BARLEY, AND RYE

Group	Type	Wheat	Barley	Rye
Sulfur-rich prolamins (30–50 kDa)	Monomeric	α/β-Gliadin, γ-gliadin	γ-Hordein	γ-Secalin
	Aggregated	Low-molecular-weight glutenin subunit	B-Hordein	—
Sulfur-poor prolamins (44–80 kDa)	—	ω-Gliadin	C-Hordein	ω-Secalin
High-molecular-weight prolamins (60–90 kDa)	—	High-molecular-weight glutenin subunit	D-Hordein	High-molecular-weight secalin

respectively (Pernollet and Mossé, 1983; Chesnut *et al.*, 1989). Because the characteristics of the glutelins of rice and the globulins of oats are similar to those of the globulins of legume seeds, they will be discussed with storage proteins of legumes (Section II,C).

2. *Structure of Genes*

The typical structure of a plant gene coding for storage proteins is shown in Fig. 1. All seed storage protein genes have the signal peptide coding region. The exons are the structural sequences that encode proteins and the introns are the intervening sequences that are removed when the primary transcript is spliced

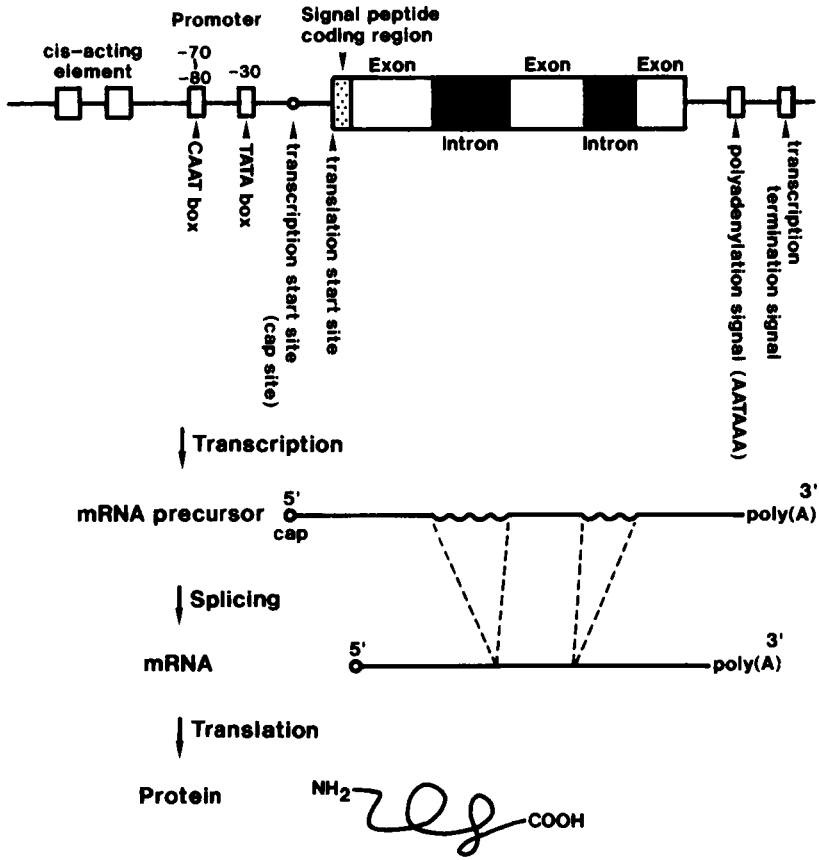


FIG. 1. Typical structure and mechanism of expression of a eukaryote gene.

to give the mature mRNA. The promoter is the DNA sequence that RNA polymerase recognizes and binds to initiate and catalyze synthesis of mRNA. Promoters of many eukaryotic genes have a sequence called a TATA box (Hogness box) around 30 base pairs (bp) upstream of the transcription start site. In all known sequences of animal genes, the consensus is

$$\mathrm{TATA}^{\mathrm{A}}_{\mathrm{T}}\mathrm{A}^{\mathrm{A}}_{\mathrm{T}}$$

The other well-conserved sequence is a CAAT box 70–80 bp upstream of the transcription start site. The consensus is

$$\mathrm{GG}^{\mathrm{C}}_{\mathrm{T}}\mathrm{CAATCT}$$

The cis-control element has the function of controlling gene expression, such as seed-specific and developmental-specific expression, as well as quantitative regulation. A sequence of poly(adenylic) acid [poly(A) tail] is added to the 3′ end of mRNA after its transcription. The consensus sequence of AATAAA is thought to be a polyadenylation signal.

Many genes and cDNAs that encode cereal seed proteins have been cloned and sequenced, as shown in Table IV. None of the genes encoding the prolamins has an intron. The rice glutelin genes have introns like those of the legume and oat globulin genes; they share similarities with the legume and oat globulins, but not with the cereal prolamins, as described in Section II,C. All the prolamin genes have a TATA box 80–130 bp upstream of the translation initiation site, but some of them have no CAAT box. With the exception of the 10-kDa zein gene, all other prolamin genes sequenced so far have DNA sequences homologous with the consensus sequence

$$\mathrm{GTGG}^{\mathrm{AAA}}_{\mathrm{TTT}}\mathrm{G}$$

which is thought to be the animal enhancer core sequence around 300 bp upstream of the translation initiation site (Table V). These sequences are called the prolamin box or the -300 element. The HMW glutenin genes have two prolamin boxes, but both of them exhibit less similarity to those of the other prolamin genes (Table V). There is a possibility that the prolamin box is a cis-control element that controls the expression of prolamin genes.

The genes encoding α-zeins have two promoter regions: the TATA box and the CAAT box 1000 bp upstream (in addition to 100 bp upstream) of the transcription initiation site (Brown *et al.*, 1986; Thompson and Larkins, 1989). At

TABLE IV

PROLAMINS FOR WHICH cDNAs AND GENES HAVE BEEN SEQUENCED

Plant	Protein	Component	Reference	
			cDNA	Gene
Maize	Zein	27 kDa	Prat *et al.* (1985, 1987)	Boronat *et al.* (1986)
		22 kDa	Marks and Larkins (1982); Heidecker and Messing (1983); Marks *et al.* (1985)	Langridge and Feix (1983); Brown *et al.* (1986)
		19 kDa	Geraghty *et al.* (1981); Pedersen *et al.* (1982); Heidecker and Messing (1983); Marks *et al.* (1985)	Pedersen *et al.* (1982); Spena *et al.* (1983); Langridge *et al.* (1985); Brown *et al.* (1986); Kriz *et al.* (1987)
		16 kDa	Prat *et al.* (1987)	
		14 kDa	Marks *et al.* (1985)	Pedersen *et al.* (1986)
		10 kDa		Kirihara *et al.* (1988)
Wheat	Gliadin	α/β	Bartels and Thompson (1983); Kasarda *et al.* (1984); García-Maroto *et al.* (1990)	Rafalski *et al.* (1984); Sumner-Smith *et al.* (1985); Reeves and Okita (1987); Anderson (1991)
		γ	Bartels *et al.* (1986)	Rafalski (1986); Sugiyama *et al.* (1986)
	Glutenin	LMW		Colot *et al.* (1987, 1989); Pitts *et al.* (1988)
		HMW	Forde *et al.* (1983)	Sugiyama *et al.* (1985); Thompson *et al.* (1985); Halford *et al.* (1987); Anderson *et al.* (1989); Flavell *et al.* (1989)
Barley	Hordein	B	Forde *et al.* (1985b)	Forde *et al.* (1985a)
		C	Forde *et al.* (1985b); Rasmussen and Brandt (1986)	
		γ		Cameron-Mills and Brandt (1988)
Rye	Secalin	γ	Kreis *et al.* (1985)	
Rice	Prolamin		Kim and Okita (1988a,b)	Kim and Okita (1988b)
		10 kDa	Masumura *et al.* (1989b); Barbier and Ishihama (1990)	
		13 kDa	Masumura *et al.* (1990)	
Oat	Avenin		Chesnut *et al.* (1989)	Shotwell *et al.* (1990)

TABLE V

SEQUENCES AND POSITIONS OF PROLAMIN BOX IN CEREAL PROLAMIN GENES

Gene	Sequence[a]	Reference
	−353	
Zein 27 kDa	CTTGACG : TGTAAA · G : TAA	Boronat *et al.* (1986)
	−341	
Zein 22 kDa	TCACATG : TGTAAA · G : GTG	Langridge and Feix (1983)
	−325	Pedersen *et al.* (1982); Brown *et*
Zein 19 kDa	ACACATG : TGTAAA · G : GTA	*al.* (1986)
	−211	
Zein 14 kDa	CTTGACA : TGTAAA · G : TTG	Pedersen *et al.* (1986)
	−297	
B1-Hordein	CGTGACA : TGTAAA · G : TGA	Forde *et al.* (1985a)
	−297	
γ-Hordein	TGTGAGA : TGTAAA · G : TGA	Cameron-Mills and Brandt (1988)
	−315	
α/β-Gliadin	TTTGAGC : TGTAAA · G : TGA	Rafalski *et al.* (1984)
	−315	
	TTTGAGC : TATAAA · G : TGA	Sumner-Smith *et al.* (1985);
	−315	Reeves and Okita (1987)
	TTTGAGT : TGTAAA · G : TGA	Sumner-Smith *et al.* (1985)
	−317	
γ-Gliadin	TTTGACA : TGTAAA · G : TGA	Rafalski (1986)
	−306	
LMW glutenin	TGTGACA : TGTAAA · G : TTA	Colot *et al.* (1989)
	−341	
	ACTCGGT : TGTAAAAG : TGA	Colot *et al.* (1989)
	−243	
	TCTGTTT : TGCAAA · G : CTC	Sugiyama *et al.* (1985); Anderson
	−243	*et al.* (1989)
HMW glutenin	TCTGTTT : TGCAAAA · : CTC	Halford *et al.* (1987)
	−243	
	ATCTTTT : TGCAAA · G : CTC	Thompson *et al.* (1985)
	−318	
	ACACTTC : TGCAAACA : ATA	Thompson *et al.* (1985);
	−313	Anderson *et al.* (1989)
	ACACTTC : TGCAAACA : ATA	Sugiyama *et al.* (1985); Anderson
	−320	*et al.* (1989)
	ACACTTC : TGCAAACA : GTA	Halford *et al.* (1987)

[a]The sequences between two colons resemble the core sequence of SV40 enhancer; a dot represents a gap. The numbers above the sequences are the positions relative to the transcription initiation site.

present the significance of the presence of two promoters in one gene is not clear, although transcription from both promoters has occurred.

3. *Structure and Conformation of Prolamins*

a. Sulfur-Rich Prolamins. The sulfur-rich prolamins have similar basic structures, with an N-terminal repetitive domain (80–130 residues) and a C-terminal unique (not repetitive) domain (150–200 residues) (Shewry and Tatham, 1990). In some cases a short unique sequence precedes the N-terminal repeat domain. The consensus motifs of the repeats are PQQPFPQ for the γ type, PQPQPFP and PQQPY for the α type, and PQQPPPFS and QQQQPVL for the aggregated type (Shewry and Tatham, 1990). The secondary structures of the two domains are predicted as follows; a β turn for the N-terminal repetitive domain and an α helix for the C-terminal unique domain, as determined by circular dichroism (CD) spectroscopy (Shewry and Tatham, 1990; Tatham *et al.*, 1990b). The C-terminal domain contains most of the cysteine residues, and the α type has poly(Q) regions.

b. Sulfur-Poor Prolamins. Among the sulfur-poor prolamins, the C-hordein of barley has been well studied. The C-hordein has unique short N-terminal and C-terminal domains and a long central repetitive domain that occupies most of the protein. The consensus motifs of the repeat are PQQPFPQQ and PQQPY (Shewry and Tatham, 1990). The latter motif is located close to the N terminus. The secondary structure of the repetitive domain is predicted to be β turns that are repeated regularly. This was confirmed by CD and nuclear magnetic resonance (NMR) spectroscopy (Shewry and Tatham, 1990). The CD and infrared (IR) spectra of the other sulfur-poor prolamins indicate that they have a structure similar to C-hordein (Shewry and Tatham, 1990).

c. HMW Prolamins. Among the HMW prolamins, HMW glutenin subunits of wheat have been characterized in detail. HMW glutenin subunits are classified into x and y types. The x types (83–88 kDa) have larger molecular masses than the y types (67–74 kDa). The true molecular masses of HMW glutenins are smaller than those (80–150 kDa) estimated by SDS–PAGE, indicating that their molecular shapes are not globular. Both types of HMW glutenin subunits have a similar basic structure, with unique N-terminal (81–104 residues) and C-terminal (42 residues) domains and a central repetitive domain. Shewry and Tatham (1990) reported the consensus motifs of the repeat peptides:

$$\text{PGQGQQ} \quad \text{and} \quad \text{GYYPTS}^{\text{P}}_{\text{L}}\text{QQ}$$

The unique N-terminal and C-terminal domains are predicted to be α-helices and the central repetitive domain consists of regularly repeated β turns (Tatham *et al.*, 1985). These predictions have been confirmed by CD spectroscopy (Field *et al.*, 1987) and CD and IR spectroscopy of synthetic peptides corresponding to the consensus sequences and that of the junction of hexa- and nona-repeat peptides (Tatham *et al.*, 1990a). Most of the cysteine residues are present in the unique N-terminal domain and one is in the unique C-terminal domain. In some cases the terminal region of the central repetitive domain has one cysteine residue (Flavell *et al.*, 1989; Goldsbrough *et al.*, 1989).

d. Zeins. The α-zeins are the main components of zeins and are rich in glutamine (25%), leucine (20%), alanine (15%), and proline (11%), but have no lysine (Shotwell and Larkins, 1989). The 22- and 19-kDa zeins consist of about 240–245 and 210–220 residues, respectively (Shewry and Tatham, 1990). Therefore, the true molecular masses of α-zeins are larger than those estimated by SDS–PAGE, indicating that these molecules are very compact. The α-zeins have similar basic structures, with unique N-terminal (about 35 residues) and C-terminal (10 or 29 residues) domains and a central repetitive domain. The repeat unit (Argos *et al.*, 1982) is composed of about 20 residues with a consensus sequence of

$$^{-2}\text{LQQ}^{\text{F}}_{\text{L}}\text{LP}^{\text{A}}_{\text{F}}\text{NQL}^{\text{AA}}_{\text{LV}}\text{ANSPAYLQQ}^{19}$$

This unit is repeated nine times in the central repetitive domain (Argos *et al.*, 1982). From these characteristics of α-zeins, Argos *et al.* (1982) proposed the structure model shown in Fig. 2. The repeat unit is predicted to form an α helix that has three polar segments on its surface (Fig. 2A). Each of the nine α helices interacts in antiparallel with the other by hydrogen bonds, using two of the three polar segments (Fig. 2B). The third polar segment is possibly utilized for intermolecular contacts (Fig. 2C). The turn regions between the antiparallel helices are rich in glutamine residues. Therefore, it is possible to form the stacked molecules as shown in Fig. 2C. This packing structure could be important for the accumulation of proteins in the protein bodies of maize (Argos *et al.*, 1982). CD spectroscopy of zeins indicates that zeins are rich in α helices (Argos *et al.*, 1982).

The β-zein (14-kDa zein) is composed of 160 residues and is rich in methionine (11%) and cysteine (4%) (Pedersen *et al.*, 1986). This protein contains a methionine-rich region but has no repetitive region, and is mainly composed of β sheets and turns (Pedersen *et al.*, 1986).

The γ-zeins (27- and 16-kDa zeins) are soluble in saline solution containing a reducing agent (Thompson and Larkins, 1989). The 27-kDa zein is composed of 204 residues. The true molecular mass of the 27-kDa zein (21.8 kDa) is much

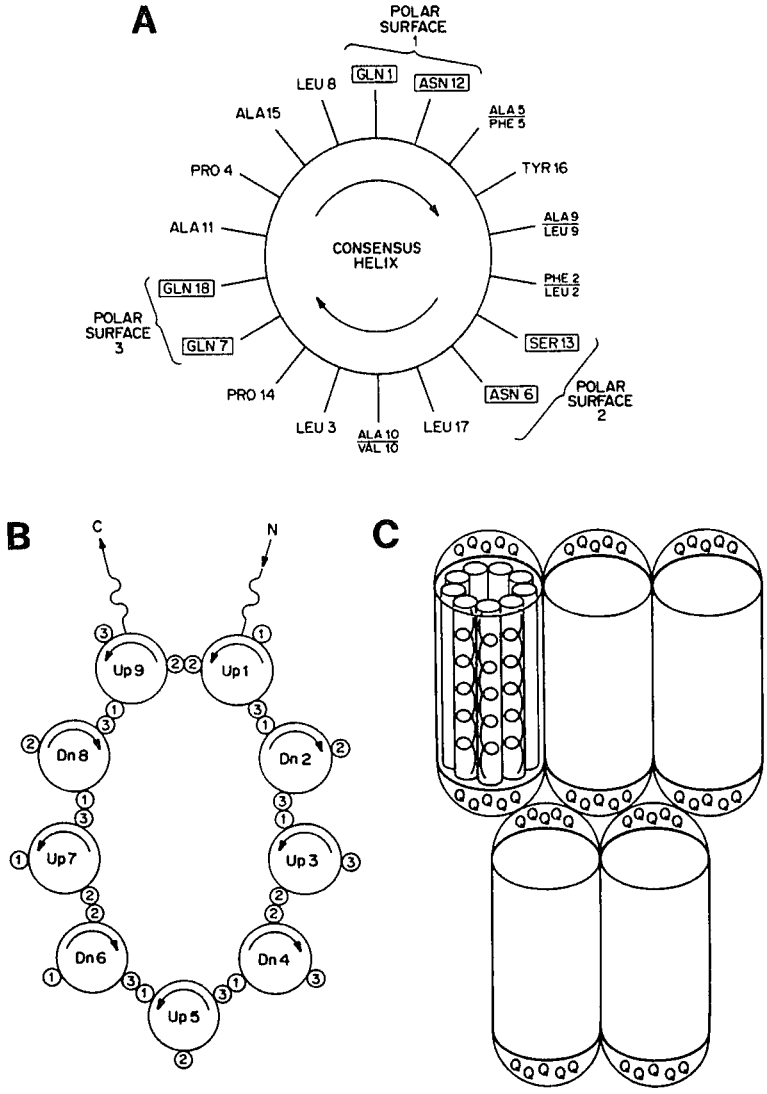


FIG. 2. Structural model for α-zeins. (A) Helical wheel for the consensus repeat sequence. The figure shows three polar regions, each composed of two amino acids. (B) A possible nine-helical zein protein structural model. The hydrogen-bonding polar residue segments (shown as small circles) are numbered as in the helical wheel (A). (C) A possible model for the arrangement of zein proteins within a plane as well as for the stacking of molecular planes. The glutamine residues (Q) in the turn regions would allow hydrogen bonding among molecules in neighboring planes. (From Argos *et al.*, 1982, with permission of the authors and publisher.)

smaller than that estimated from SDS–PAGE, indicating that its conformation is different from those of α-zeins. The 27-kDa zein has unique N-terminal (11 residues) and C-terminal (145 residues) domains and a short central repetitive domain (48 residues). The consensus motif of the repeat if PPPVHL. The 16-kDa zein has an amino acid sequence and structure similar to those of the 27-kDa zein (Prat *et al.*, 1987). Both γ-zeins are rich in cysteine (Prat *et al.*, 1987).

The δ-zein (10-kDa zein), like β-zein, is rich in sulfur-containing amino acids, especially methionine (22%) (Kirihara *et al.*, 1988). However, δ-zein exhibits no homology with the other zeins.

e. Other Prolamins and Their Relationship among Prolamins. The primary structures of 13- and 10-kDa prolamins of rice and avenin of oats were predicted from the nucleotide sequences of their cDNAs and genes. They exhibit homology with each other and with α-type and γ-type prolamins of wheat, barley, and rye (Chesnut *et al.*, 1989; Masumura *et al.*, 1990).

Kreis and Shewry (1989) compared the primary structure of various prolamins and observed three homologous regions (A, B, and C) among HMW prolamins, sulfur-rich prolamins, sulfur-poor prolamins, β-zein, and γ-zeins. The same authors also observed the same homology in some enzyme inhibitors, 2S globulins, and 2S albumins. The 13- and 10-kDa prolamins of rice and avenin of oats also exhibit homology with region B. Therefore, these proteins could have been derived from the same ancestral gene (Kreis and Shewry, 1989; Shewry and Tatham, 1990). The α-zeins have no homology with regions A, B, and C.

f. Solubility of Prolamins. Kreis and Shewry (1989) suggested that the important property shared by prolamins is their insolubility in aqueous salt solutions. Their solubility in aqueous alcohol is variable, i.e., some prolamins (LMW and HMW prolamins, etc.) are soluble in aqueous alcohol only in the presence of a reducing agent. Moreover, the 27-kDa zein is soluble in aqueous salt solution in the presence of a reducing agent. The same authors attributed the insolubility of prolamins in aqueous salt solutions to three structural features: (1) the presence of repetitive sequences that are rich in aromatic amino acids and glutamine (the amide side chains of glutamines form hydrogen bonds, resulting in a hydrophobic nature), (2) the presence of a methionine-rich region having a hydrophobic nature, and (3) the presence of interchain disulfide bonds.

C. STORAGE PROTEINS OF LEGUMES

1. Classification

Globulins are the dominant storage proteins in legume seeds and account for 50–90% of seed proteins. The globulins of legume seeds are classified into two

types according to their sedimentation coefficients: 7S globulins (7.1–8.7S) and 11S globulins (10.1–14S). The content of sulfur-containing amino acids of 11S globulin is higher than that of the 7S globulin (Derbyshire *et al.*, 1976). The seeds of soybean (*Glycine max*), field bean (horse bean, broad bean; *Vicia faba*), pea (*Pisum sativum*), and other legumes contain the two types of globulins, although those of french bean (*Phaseolus vulgaris*) and winged bean (*Psophocarpus tetragonolobus*) contain only or predominantly 7S globulins. Even in plant species containing both globulins, the ratio of 11S to 7S globulins varies among cultivars from 0.5 to 1.7 in soybean, 0.2 to 1.5 in pea, and 0.3 to 0.5 in field bean (Wright, 1987).

The 7S globulin is a glycoprotein with a molecular mass of 150–200 kDa and is generally composed of three subunits with molecular masses of 40–70 kDa (Pernollet and Mossé, 1983). The typical 7S globulins are β-conglycinin of soybean, vicilins of pea and field bean, and phaseolin of french bean (Pernollet and Mossé). β-Conglycinin is composed of α, α′, and β subunits. Pea seeds contain another 7S globulin, convicilin, which has a molecular mass of ~290 kDa and is composed of subunits with a molecular mass of 71 kDa (Croy *et al.*, 1980). This protein is not glycosylated but has properties similar to vicilin (Croy *et al.*, 1980), although it is not yet clear whether this protein is a tetramer or a trimer. A protein similar to convicilin is present in field bean (Croy *et al.*, 1980). Soybean seeds contain another 7S globulin, a basic 7S globulin that is a methionine- and cysteine-rich glycoprotein (Yamauchi *et al.*, 1984; Kagawa *et al.*, 1987; Kagawa and Hirano, 1989). However, this protein is not related to 11S or 7S globulins.

The 11S globulin is a simple protein with a molecular mass of 300–400 kDa and is composed of six subunits with molecular masses of 50–60 kDa. The typical 11S globulins are glycinin of soybean, legumins of pea and field bean, and arachin of peanut (Pernollet and Mossé, 1983). These 11S globulins are widely distributed in seeds of nonlegume plants, such as sunflower (*Helianthus annuus*), cotton (*Gossypium hirsutum*), rape (*Brassica napus*), pumpkin (*Cucurbita pepo*), sesame (*Sesamum indicum*), and oats (Shotwell and Larkins, 1989). The glutelin of rice has a structure similar to those of 11S globulins of the legume and nonlegume seeds, as is described below. Therefore, 11S globulins of the nonlegume and oat seeds and glutelin of rice are also mentioned in this section.

2. *Structure of Genes*

a. 7S Globulins. Many genes and cDNAs that encode 7S globulins have been cloned and sequenced (Table VI). With the exception of one of the convicilin genes (Newbigin *et al.*, 1990), all of the 7S globulin genes determined so far have six exons and five introns (Table VII). The size (base pairs) of each exon

TABLE VI

7S GLOBULINS FOR WHICH cDNAs AND GENES HAVE BEEN SEQUENCED

Plant	Protein	Subunit	Reference	
			cDNA	Gene
Field bean	Phaseolin	α	Slighton *et al.* (1985)	Anthony *et al.* (1990)
		β	Slighton *et al.* (1985)	Slighton *et al.* (1983)
Soybean	β-Conglycinin	α	Schuler *et al.* (1982a,b); Sebastiani *et al.* (1990); Coates *et al.* (1985)	Schuler *et al.* (1982a)
		α′	Schuler *et al.* (1982a,b)	Schuler *et al.* (1982a); Doyle *et al.* (1986)
		β		Harada *et al.* (1989)
Pea	Vicilin	47 kDa	Lycett *et al.* (1983)	
		50 kDa	Lycett *et al.* (1983); Spencer *et al.* (1983)	Higgins *et al.* (1988)
		68 kDa	Domoney and Casey (1990)	
	Convicilin		Casey *et al.* (1984); Newbigin *et al.* (1990)	Bown *et al.* (1988) Newbigin *et al.* (1990)
Field bean	Vicilin		Bassüner *et al.* (1987)	Weschke *et al.* (1987)
Cotton	α-Globulin		Chlan *et al.* (1987)	Chlan *et al.* (1987)

and intron exhibits a rough homology among the 7S globulin genes (Table VII). There is a homology in the nucleotide sequences of the exon and intron junctions (Table VIII). All the genes have a TATA box 30–40 bp upstream of the transcription initiation site (Table VII) and the consensus sequence is

$$\text{TATAAATA}\begin{matrix}\text{T}\\\text{C}\end{matrix}$$

Some of the genes have a CAAT box and the DNA sequence of homology with the animal enhancer core sequence. All of the genes, except the β-conglycinin β-subunit gene, have a vicilin box 120–140 bp upstream of the transcription initiation site (Gatehouse *et al.*, 1986). The vicilin box is a highly conserved region among the 7S globulin genes, but not in any other genes. The conservativity is very high among the 7S globulin genes (Table IX). The vicilin box may regulate the expression of the 7S globulin genes (Gatehouse *et al.*, 1986). Goldberg (1986) observed another conserved sequence, the CACA box, from the comparison of the DNA sequences of soybean protein genes. The consensus sequence of the CACA box is

$$\begin{matrix}\text{A} & & \text{A}\\\text{TAACACA}&&\begin{matrix}\text{A}\\\text{G}\end{matrix}\text{T}\\\text{C} & & \text{G}\end{matrix}$$

TABLE VII

CONSTRUCTION OF 7S GLOBULIN GENES

Gene	Vicilin box[a] (bp)	TATA box[a] (bp)	Signal peptide (residues)	Exon[b] I	Intron I	Exon II	Intron II	Exon III	Intron III	Exon IV	Intron IV	Exon V	Intron V	Exon VI	Reference
Phaseolin β	−138	−32	(24)	243	72	191	88	81	124	231	128	259	103	188	Doyle *et al.* (1986)
β-Conglycinin α′	−120	−32	(25)	832	203	233	87	81	85	297	115	280	132	194	Doyle *et al.* (1986)
β-Conglycinin β	x[c]	−31	(23)	301	269	176	130	81	104	285	142	280	99	194	Harada *et al.* (1989)
Pea vicilin	−131	−32	(27)	304	342	176	170	81	103	324	240	313	133	175	Higgins *et al.* (1988)
Field bean vicilin 1	−131	−34	(27)	304	145	176	171	81	100	314	250	308	109	186	Weschke *et al.* (1987)
Convicilin A	−137	−38	(28)	661	151	176	103	75	103	324	88	283	97	197	Bown *et al.* (1988)
	−137	−38	(29)	805	151	1016	—	—	—	—	—	—	—	—	Newbigin *et al.* (1990)

[a]Position from the transcription initiation site.

[b]Values are given in base pairs for all exons and introns.

[c]Not present.

TABLE VIII

NUCLEOTIDE SEQUENCES AROUND EXON AND INTRON JUNCTIONS OF 7S GLOBULIN GENES[a]

Intron	Phaseolin β	β-Conglycinin α′	β-Conglycinin β	Pea vicilin	Field bean vicilin	Convicilin	Consensus sequences
I	GTG/gta—cag/GGA	ACG/gtt—cag/GGA	GCG/gtt—cag/GGA	GTG/gta—cag/GAA	GTG/gta—cag/GAA	ATG/gta—cag/GGA	AC a G G/gt —cag/G A GT t A
II	CAT/gta—tag/GAA	GAG/gta—cag/AGT	GAT/gta—tag/GAT	CAG/gta—cag/TCT	CAG/gta—cag/TCT	GAG/gta—cag/GCT	C G t GA A gta— ag/ T G T c TC
III	AAT/gta—tag/AGC	GAC/gta—tag/ACC	CAT/gta—tag/AGC	AAT/gta—tag/ACT	AAT/gta—tag/ACC	AAT/gta—tag/ACT	GC AAT/gta—tag/A CT
IV	GAG/gta—tag/GGA	GAA/gta—tag/GGA	GAA/gta—tag/GGA	GAG/gta—tag/GGA	CAG/gta—tag/GGA	AAG/gta—tag/GGA	G GA /gta—tag/GGA A
V	CAG/gta—aag/GTA	CAG/gta—aag/GTT	CAG/gta—aag/GCG	CAG/gta—aag/GCG	CAG/gta—aag/GTG	CAG/gta—aag/GAT	TG CAG/gta—aag/G CT

[a]The references are the same as those listed in Table VII.

TABLE IX

SEQUENCES AND POSITIONS OF VICILIN BOX IN LEGUME 7S GLOBULIN GENES

Gene	Sequence[a]	Reference
	−120	
β-Conglycinin α′	CC : GCCACCTCATTTTTGTTTATTTCAACACCCGTCAAACTGCAT : CC	Doyle *et al.* (1986)
	−138	
Phaseolin β	CC : GCCACCTCAATTTC · TTCACTTCAACACACGTCAACCTGCAT : AT	Doyle *et al.*(1986)
	−138	
Phaseolin α	CC : GCCACCTCAATTTC · TTCACTTCAACACACGTGAACCTGCAT : AT	Anthony *et al.* (1990)
Pea vicilin B	TT : GCCACCTCAATTTTGTACATTTCAACACACGTCCATATGCAT : GG	Gatehouse *et al.* (1986)
Pea vicilin J	TA : GCCACCTCAATTTTGTTCATTTCTACACTAGTCAACATGCAT : GG	Gatehouse *et al.* (1986)
	−131	
Pea vicilin	TT : GCCACTTCAATTTTGTACATTTTAACACACGTCCATATGCAT : GG	Higgins *et al.* (1988)
	−131	
Field bean vicilin 1	TT : GCCACCTCAATTTTGTACATTTCAACACACGTCCATATGCAT : GG	Weschke *et al.* (1987)
	−137	
Convicilin	TT : GCCACCTCTATTTTGTTCATTTCAACACTCGTCAAGTTACAT : GA	Bown *et al.* (1988)
	−137	
Convicilin	TT : GCCACCTCTATTTTGTTCATTTCAACACTCGTCATGCTACAT : GG	Newbigin *et al.* (1990)

[a]The sequences between two colons are vicilin boxes. The numbers above the sequences are the positions relative to the transcription initiation site. A dot in the sequence represents the gap.

The homologous sequence of this element is present in many 7S globulin genes (Table X). Because this sequence is present in many genes encoding seed proteins (Table X), it is possible that it could play some role in regulating the expression of seed protein genes (Goldberg, 1986).

b. 11S Globulins. Many genes and cDNAs that encode 11S globulins of legume and nonlegume seeds have been cloned and sequenced (Table XI). Most of 11S globulin genes determined so far have four exons and three introns, but the genes encoding the B-type subunit of pea and field bean lack intron I and the genes of rape and sunflower lack intron III (Table XII). However, there is a rough homology in the structure among each gene (Table XII) and a homology in the nucleotide sequences around the exon and intron junctions (Table XIII). The characteristics of TATA, CAAT, and CACA boxes and the animal enhancer core sequence in the 5′ upstream regions of the 11S globulin genes are similar to those of the 7S globulin genes. Sequence comparison of the 11S globulin genes revealed a highly conserved sequence of a legumin box 100–130 bp upstream of the transcription initiation site (Tables XII and XIV) (Bäumlein *et al.*, 1986). The legumin box is observed only in the 11S globulin genes, and all of the 11S globulin genes of legume seeds examined so far have this box. However, of the nonlegume seed 11S globulin genes, only the rape cruciferin gene has the legumin box (Table XII). The position (248 bp upstream of the transcription initiation site) of the legumin box of the rape cruciferin gene is different from the positions of the legumin box of the legume seed 11S globulin

TABLE X

SEQUENCES AND POSITIONS OF CACA BOX IN LEGUME PROTEIN GENES

Protein	Gene	Position	Sequence	Reference
7S globulin	Phaseolin α	−198	AAACACAAT	Anthony *et al.* (1990)
		−117	CAACACACG	
	Phaseolin β	−198	AAACACATT	Doyle *et al.* (1986)
		−117	CAACACACG	
	β-Conglycinin α′	−387	AAACACAAT	Doyle *et al.* (1986)
	β-Conglycinin β	−104	CAACACAGT	Harada *et al.* (1989)
	Pea vicilin	−109	TAACACACG	Higgins *et al.* (1988)
	Field bean vicilin	−109	CAACACACG	Weschke *et al.* (1987)
	Convicilin	−698	GAACACATA	Newbigin *et al.* (1990)
11S globulin	Glycinin $A_{1a}B_{1b}$	−428	CAACACAAT	Sims and Goldberg (1989)
	Glycinin A_2B_{1a}	−130	TAACACACA	Thanh *et al.* (1989)
	Pea legumin	−550	TAACACAAG	Gatehouse *et al.* (1988)
	Rice glutelin	−185	CAACACAAT	Takaiwa *et al.* (1987a)

TABLE XI

11S GLOBULINS FOR WHICH cDNAs AND GENES HAVE BEEN SEQUENCED

Plant	Protein	Subunit	Reference	
			cDNA	Gene
Soybean	Glycinin	$A_{1a}B_{1b}$	Negoro *et al.* (1985); Utsumi *et al.* (1987b)	Sims and Goldberg (1989)
		$A_{1b}B_2$		Cho and Nielsen (1989)
		A_2B_{1a}	Marco *et al.* (1984); Momma *et al.* (1985b); Utsumi *et al.* (1987a)	Marco *et al.* (1984); Fukazawa *et al.* (1987a); Thanh *et al.* (1989); Kitamura *et al.* (1990)
		A_3B_4	Fukazawa *et al.* (1985); Scallon *et al.* (1985)	Nielsen *et al.* (1989)
		$A_5A_4B_3$	Momma *et al.* (1985a); Scallon *et al.* (1985)	Scallon *et al.* (1987); Nielsen *et al.* (1989)
Pea	Legumin	—	Croy *et al.* (1982); Lycett *et al.* (1984b); Domoney *et al.* (1986)	
Pea	Legumin	A		Lycett *et al.* (1984a); Rerie *et al.* (1990)
		B, C		Lycett *et al.* (1985)
		J, K		Gatehouse *et al.*(1988)
		(Pseudo)		Bown *et al.* (1985)
Field bean	Legumin	A	Wobus *et al.* (1986); Schlesier *et al.* (1990)	
		B	Wobus *et al.* (1986)	Bäumlein *et al.* (1986); Heim *et al.* (1989)
Oat	12S globulin	—	Walburg and Larkins (1986); Shotwell *et al.* (1988)	Schubert *et al.* (1990); Shotwell *et al.* (1990)
Rice	Glutelin	—	Takaiwa *et al.* (1986, 1987b, 1989); Higuchi and Fukazawa (1987); Wang (1987); Masumura *et al.* (1989a); Okita *et al.* (1989); Wen *et al.* (1989)	Takaiwa *et al.* (1987a) Okita *et al.* (1989)
Sunflower	Helianthinin	—		Vonder Haar *et al.* (1988); Bogue *et al.* (1990)
Arabidopsis	12S globulin	—		Pang *et al.* (1988)
Cotton	β-Globulin	—	Chlan *et al.* (1986)	
Rape	Cruciferin	—	Simon *et al.* (1985); Rödin *et al.* (1990); Sjödahl *et al.* (1991)	Ryan *et al.* (1989)
Pumpkin	11S globulin	β	Hayashi *et al.* (1988)	

TABLE XII

CONSTRUCTION OF 11S GLOBULIN GENES

Protein	Legumin box[a] (bp)	TATA box[a] (bp)	Signal peptide (residues)	ExonI[b]	IntronI	ExonII	IntronII	ExonIII	IntronIII	ExonIV	Reference
Glycinin $A_{1a}B_{1b}$	−117	−32	(19)	286	328	254	291	558	381	387	Sims and Goldberg (1989)
Glycinin A_2B_{1a}	−116	−32	(18)	277	238	254	292	537	624	387	Thanh *et al.* (1989)
Glycinin $A_{1b}B_2$	−117	−32	(19)	286	617	245	312	525	439	387	Cho and Nielsen (1989)
Pea legumin A	−117	−33	(21)	286	88	251	88	627	99	387	Lycett *et al.* (1984a)
Pea legumin A-type	−127	−32	(22)	279	88	251	89	633	85	387	Rerie *et al.* (1990)
Arabidopsis CRA1	?[c]	−56[d]	?	301	126	266	96	447	115	402	Pang *et al.* (1988)
Arabidopsis CRB	X[e]	−62[d]	?	283	210	263	311	429	239	390	Pang *et al.* (1988)
Glutelin 1-2	X	−32	(24)	331	89	275	103	480	83	411	Takaiwa *et al.* (1987a)
Oat 12S globulin	X	−32	?	328	117	271	122	512	100	435	Schubert *et al.* (1990)
	X	−28	(24)	328	117	275	126	516	100	435	Shotwell *et al.* (1990)
Pea legumin J	−102	−27	(22)	549	—	—	138	585	99	375	Gatehouse *et al.* (1988)
Field bean legumin B4	−107	−32	(22)	549	—	—	95	528	100	375	Bäumlein *et al.* (1986)
Cruciferin	−248	−36	(23)	282	228	362	467	819	—	—	Ryan *et al.* (1989)
Helianthinin	X	−27	(20)	288	99	258	80	933	—	—	Vonder Haar *et al.* (1988)

[a]Position from the transcription initiation site.

[b]Values are given in base pairs for all exons and introns.

[c]Not determined.

[d]Position from the translation initiation site.

[e]Not present.

TABLE XIII

NUCLEOTIDE SEQUENCES AROUND JUNCTIONS OF EXONS AND INTRONS OF 11S GLOBULIN GENES

Gene	Intron I	Intron II	Intron III	Reference
Glycinin $A_{1a}B_{1b}$	AAG/gtc—tag/GTA	AGG/gtg—cag/AGA	AAG/gta—cag/AAT	Sims and Goldberg (1989)
Glycinin A_2B_{1a}	AAC/gtc—tag/GTA	AGG/gtg—cag/AGA	AAG/gta—cag/AAT	Thanh *et al.* (1989)
Glycinin $A_{1b}B_2$	AAG/gtt—tag/GTA	AGG/gta—cag/AGA	AAG/gta—cag/AAT	Cho and Nielsen (1989)
Pea legumin A	AAG/gtt—cag/GTA	AGG/gtg—cag/AGA	AAA/gta—cag/AAT	Lycett *et al.* (1984a); Rerie *et al.* (1990)
Arabidopsis CRA1	AGG/gta—tag/GAC	AGG/gta—aag/CCA	CAA/gta—tag/AAC	Pang *et al.* (1988)
Arabidopsis CRB	ACG/gtg—tag/GAA	AGA/gta—cag/CCA	AAA/gta—cag/AAC	Pang *et al.* (1988)
Glutelin 1-2	AAG/gtt—cag/GGA	AGG/gta—tag/GAT	CAG/gta—cag/AAT	Takaiwa *et al.* (1987a)
Oat 12S globulin	AAG/gtt—tag/GTA	AAG/gta—tag/GAG	CAG/gta—cag/AAT	Schubert *et al.* (1990); Shotwell *et al.* (1990)
Pea legumin J	—	AGA/gta—cag/GTA	AGG/gta—cag/AAT	Gatehouse *et al.* (1988)
Field bean legumin B4	—	AGA/gta—cag/GTA	AGG/gta—cag/AAT	Bäumlein *et al.* (1986)
Cruciferin	AAG/gta—tag/GAG	AGG/gta—tag/CCA	—	Ryan *et al.* (1989)
Helianthinin	GAG/gta—cag/GGG	AGG/gta—cag/AGA	—	Vonder Haar *et al.* (1988)
Consensus sequences	$AAG/gt^{a}_{t}—^{c}_{t}ag/G^{G}_{A}{}^{A}_{G}$ T	G g c A AG /gt — ag/GCA A a a CT	$AAG_{/gta—cag/AA}{}^{T}_{C}$ CGA	—

TABLE XIV

SEQUENCES AND POSITIONS OF LEGUMIN BOXES IN 11S GLOBULIN GENES

Gene	Sequence[a]	Reference
	−117	
Glycinin $A_{1a}B_{1b}$	CT : TCCATAGCCATGCATACTGAAGAATGTC : TC	Sims and Goldberg (1989)
	−116	
Glycinin A_2B_{1a}	CT : TCCATAGCCATGCATACTGAAGAATGTC : TC	Thanh *et al.* (1989)
	−117	
Glycinin $A_{1b}B_2$	GT : TCCATAGCCATGCATACTGAAGAATGTC : TC	Cho and Nielsen (1989)
	−117	
Pea legumin A	CT : TCCATAGCCATGCAAGCTGCAGAATGTC : CA	Lycett *et al.* (1984a)
	−127	
Pea legumin A	CT : TCCAAAACCATGCAAGATGAAGAATGTC : GA	Rerie *et al.* (1990)
	−117	
Pea legumin B	CT : TCCATAGCCATGCAAGCTGCAGAATGTC : CA	Lycett *et al.* (1985)
	−117	
Pea legumin C	CT : TCCATAGCCATGCAAGCTGCAGAATGTC : CA	Lycett *et al.* (1985)
	−102	
Pea legumin J	AC : TCCATAGCCATGCATGCTGAACAATGTC : AT	Gatehouse *et al.* (1988)
	−107	
Field bean legumin B4	AC : TCCATAGCCATGCATGCTGAAGAATGTC : AC	Bäumlein *et al.* (1986)
	−248	
Cruciferin	TT : CAATAAGCCATGCAAATTGAGATGTGTC : AA	Ryan *et al.* (1989)

[a]The sequences between two colons are the legumin boxes. The numbers above the sequences are the positions relative to the transcription initiation site.

genes, and, moreover, there is less homology of the nucleotide sequence between the cruciferin gene and the legume genes (Table XIV). Therefore, there is a possibility that the legumin box could regulate the expression of the legume seed 11S globulin genes.

3. *Structure and Conformation of Proteins*

a. 7S Globulins. The 7S globulins are generally trimers. However, vicilin of pea has subunits with molecular masses of 33, 19, 16, 13.5, and 12.5 kDa, which are derived from posttranslational proteolysis of a 50-kDa precursor

(Gatehouse *et al.*, 1982, 1983; Scholz *et al.*, 1983; Spencer *et al.*, 1983). Field bean vicilin is also processed similarly (Wright, 1987).

The β-conglycinin of soybean is composed of three kinds of subunits, α, α′, and β (Thanh and Shibasaki, 1976), which have molecular masses of 57–68, 57–72, and 42–52 kDa, respectively. This protein exhibits molecular heterogeneity, and seven molecular species have been identified as β_3, $\alpha'\beta_2$, $\alpha\beta_2$, $\alpha\alpha'\beta$, $\alpha_2\alpha'$, and α_3 (Thanh and Shibasaki, 1978; Yamauchi *et al.*, 1981). The β-conglycinin subunits exhibit association–dissociation depending on pH and ionic strength of the solution (Thanh and Shibasaki, 1979).

Phaseolin of french bean is composed of three kinds of subunits, α, β, and γ, with molecular masses of 43–53 kDa (Hall *et al.*, 1977; Brown *et al.*, 1981). The α and β types consist of 411–412 and 397 residues.

Electron microscopy of phaseolin, β-conglycinin, and pea vicilin indicated that they appear as flat disks about 8.5 nm in diameter and about 3.5 nm thick (Tulloch and Blagrove, 1985). Crystallization of phaseolin was successfully achieved by Suzuki *et al.* (1983) and the determination of its three-dimensional structure at 3 Å resolution was performed as shown in Fig. 3 (Lawrence *et al.*, 1990). Phaseolin polypeptide is composed of two structurally similar units, each made up of a β barrel having a "jelly roll" folding topology and an α-helix domain (Fig. 3A). The phaseolin trimer stereo pair is shown in Fig. 3B. The CD and optical rotary dispersion (ORD) spectroscopic patterns suggest that the 7S globulin is composed principally of β sheets with a low content of α helices (Wright, 1987). This is also consistent with phaseolin of X-ray analysis data (Lawrence *et al.*, 1990).

Wright (1987, 1988) aligned the amino acid sequences to maximize the homology among the 7S globulins. He observed 46% absolute homology between phaseolin and pea vicilin. If conservative substitutions (e.g., Arg and Lys, Gly and Pro) are considered, the homology increases to 65%. Similarly, pea vicilin and the α′ subunit of β-conglycinin share 53 and 72% homology, respectively. From such comparisons, Wright (1988) suggested that they comprised a series of alternating conserved and variable domains, that is, one extension, four conserved, and five variable regions (Table XV). Conserved domains I and III resemble each other. Gibbs *et al.* (1989) suggested that the 7S globulin gene evolved from the duplication of one ancestral gene. These observations are consistent with the results of X-ray analysis of phaseolin (Lawrence *et al.*, 1990).

b. 11S Globulins. The 11S globulins are hexamers in which each subunit is composed of an acidic polypeptide (acidic p*I*) with a molecular mass of 30–40 kDa and a basic polypeptide (basic p*I*) with a molecular mass of 18–22 kDa. The acidic and basic polypeptides are linked together by a disulfide bond (Badley *et al.*, 1975; Kitamura *et al.*, 1976; Mori *et al.*, 1979; Staswick *et al.*, 1981, 1984). Initially a single polypeptide precursor is synthesized and then processed

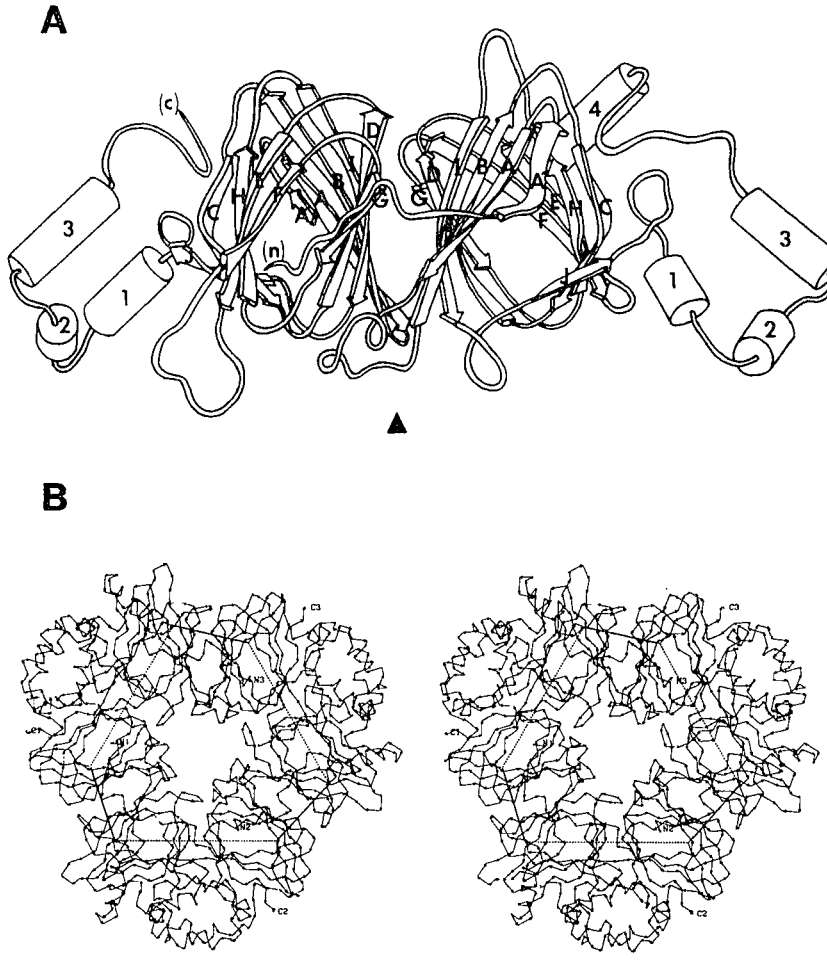


FIG. 3. (A) Schematic diagram of the phaseolin polypeptide. Plane arrows and cylinders are β sheets and α helices, respectively. The N and C termini are labeled (n and c). The view is down the molecular threefold axis (indicated as a solid triangle) and from the center of the tetramer outward. (B) Stereo pair of the phaseolin trimer. (From Lawrence *et al.*, 1990, with permission of the authors and publisher.)

posttranslationally to form the acidic (A) and the basic (B) polypeptides (Turner *et al.*, 1981, 1982; Barton *et al.*, 1982; Chrispeels *et al.*, 1982a). The fundamental structure of the 11S globulin is 6(A–B).

Rice glutelin is a macromolecule composed of disulfide-bonded subunits, although the structures of the constituent subunits are similar to those of 11S

TABLE XV

DOMAIN STRUCTURE OF 7S GLOBULINS[a]

Domain	Aligned residue no.[b]	Characteristics
Extension (variable)	—	Present in α-globulin B, convicilin, and α and α′ subunits of β-conglycinin
Variable I	1–34	
Conserved I	35–125	Homology region A (76–120); residues 36–65 homologous with residues 290–319; residues 74–111 homologous with residues 317–354
Variable II	126–155	Tandem repeat in β-conglycinin α
Conserved II	156–225	Homology region B (198–223)
Variable III	226–249	—
Conserved III	250–354	Homology region C (306–354); residues 290–319 homologous with residues 36–65; residues 317–354 homologous with residues 74–111
Variable IV	355–386	—
Conserved IV	387–499	Homology region D (391–421)
Variable V	500–525	—

[a]From Wright (1988), with permission of the author and publisher.
[b]Containing gaps.

globulin subunits. Glutelin has eight cysteine residues (six on the acidic polypeptide and two on the basic one) (Takaiwa *et al.*, 1987a,b). Since some of the positions of the seven cysteine residues in the primary structure are not equivalent to those of legume 11S globulins, there is a possibility that such cysteine residues contribute to the formation of a macromolecule.

Five subunits of glycinin have been identified, i.e., $A_{1a}B_{1b}$, $A_{1b}B_2$, A_2B_{1a}, A_3B_4, and $A_5A_4B_3$ (Nielsen, 1984, 1985). The number and the position of the disulfide bond between the acidic and basic polypeptides of four of the five subunits were determined by Staswick *et al.* (1984). Only one disulfide bond was found to be involved in linking the acidic and basic polypeptides of each subunit, and they were in analogous positions (Staswick *et al.*, 1984). That is, the cysteine residue at position 7 from the N terminus of the basic polypeptide and that at around position 90 from the N terminus of the acidic polypeptide form a disulfide bond (Staswick *et al.*, 1984). All of the 11S globulins and glutelin subunits determined so far have cysteine residues in homologous positions (Table XVI). These facts suggest that the disulfide bond between the acidic and the basic polypeptides of 11S globulin subunits is located in a homologous

position regardless of the origin of the 11S globulins. The processing site between the acidic and the basic polypeptides of all of the 11S globulin subunits is asparagine–glycine (Table XVI). Therefore, all of the 11S globulin subunits have Asn at the C terminus of the acidic polypeptide and glycine at the N terminus of the basic polypeptide. However, there is one exception, ginnacin of *Ginkgo biloba;* the N terminus of its basic polypeptide is asparagine and it has cysteine at position 7 (Bilani *et al.*, 1990). Furthermore, Staswick *et al.* (1984) suggested that one of the internal disulfide bonds within the acidic polypeptide, A_2, is Cys 10–Cys 51. All of the 11S globulin subunits known so far have a cysteine at residue 33 downstream from the cysteine residue in the neighborhood of the N-terminus of the acidic polypeptide, and the amino acid sequences around these cysteine residues are well conserved (Table XVII). These facts, together with the result obtained by Staswick *et al.* (1984), suggest that the cysteine residues listed in Table XVII could be involved in an internal disulfide bond within the acidic polypeptide (Wright, 1987), and that in the case of glycinin A_2B_{1a}, the intradisulfide bond is Cys 10–Cys 43, not Cys 10–Cys 51.

The $A_5A_4B_3$ subunit of glycinin has an unusual structure (Momma *et al.*, 1985a). This subunit is synthesized as a single polypeptide similarly to the others, but the acidic polypeptide is cleaved to produce A_5 (97 residues) and A_4 (257 residues) polypeptides. An A_5 polypeptide is derived from the N-terminal region of the acidic polypeptide region. The cleaved site between A_5 and A_4 is asparagine 97 and arginine 98. The disulfide bond between the acidic and the basic polypeptides of $A_5A_4B_3$ is between A_5 and B_3.

Vicia faba legumin (Utsumi *et al.*, 1980b) and soybean glycinin (Mori *et al.*, 1981) exhibit polymorphism of their subunit compositions among the cultivars. The 11S globulins of *V. faba* and soybean cultivars were, respectively, classified into three and five groups according to the subunit composition as determined by isoelectric focusing. They also exhibit molecular heterogeneity, and hence the occurrence of molecular species with different subunit compositions and molecular weights (Utsumi and Mori, 1980, 1981; Utsumi *et al.*, 1981). Similar phenomena have been described for pea (Thompson *et al.*, 1978), peanut (Tombs, 1963, 1965; Tombs and Lowe, 1967), and *Lupinus angustifolius* (Gillespie and Blagrove, 1975; Blagrove and Gillespie, 1978). Therefore, it is not likely that the 11S globulins exist as a unique molecular species.

The primary structure of the 11S globulins is highly conserved among the plant species (Nielsen *et al.*, 1989). According to the extent of the homology, the 11S globulin subunits are classified into two groups: group I ($A_{1a}B_{1b}$, $A_{1b}B_2$, and A_2B_{1a} of glycinin, pea legumin A, etc.) and group II (A_3B_4 and $A_5A_4B_3$ of glycinin, pea legumin J, broad bean legumin B_4, etc.) (Nielsen *et al.*, 1989). Such a homology of the primary structure of the 11S globulins makes possible the exchange of the acidic and basic polypeptides of *V. faba* legumin, soybean

TABLE XVI

AMINO ACID SEQUENCES AROUND CYSTEINE RESIDUES ASSUMED TO BE INVOLVED IN INTERCHAIN DISULFIDE BOND[a]

11S globulin	Acidic polypeptide	Basic polypeptide	Reference
	88	↓ 7	
Glycinin $A_{1a}B_{1b}$	–I Y P G C P S T F–	–R N G I D E T I C T M R L–	Utsumi *et al.* (1987b); Sims and Goldberg (1989)
	88		
Glycinin $A_{1a}B_{1b}$	–I Y P G C S S T F–	–R N G I D E T I C T M R L–	Negoro *et al.* (1985)
	88		
Glycinin $A_{1b}B_2$	–I F P G C P S T F–	–R N G I D E T I C T M R L–	Cho and Nielsen (1989)
	86		
Glycinin A_2B_{1a}	–I F P G C P S T Y–	–R N G I D E T I C T M R L–	Momma *et al.* (1985b); Utsumi *et al.* (1987a); Thanh *et al.* (1989)
	85		
Glycinin A_3B_4	–A F P G C P E T F–	–R N G V E E N I C T M K L–	Fukazawa *et al.* (1985)
	85		
Glycinin $A_5A_4B_3$	–A I P G C P E T F–	–R N G V E E N I C T L K L–	Momma *et al.* (1985a)
	86		
Pea legumin A	–V E P G C P E T F–	–D N G L E E T V C T A K L–	Lycett *et al.* (1984a); Rerie *et al.* (1990)
	87		
Pea legumin J	–S F P G C P E T Y–	–K N G L E E T I C S A K I–	Gatehouse *et al.* (1988)
	86		
Field bean legumin A	–V F P S C P E T F–	–D N G L E E T V C T A K L–	Schlesier *et al.* (1990)
	87		
Field bean legumin B4	–T L P G C P Q T Y–	–R N G L E E T I C S L K I–	Bäumlein *et al.* (1986)
	97		
Oat 12S globulin	–T F P G C P A T F–	–F N G L E E N F C S L E A–	Shotwell *et al.* (1988)
	112		
Arabidopsis CRA1[b]	–V I P G C A E T F–	–G N G L E E T I C S A R C–	Pang *et al.* (1988)
	106		
Arabidopsis CRB[b]	–V I P G C A E T F–	–A N G L E E T L C T M R C–	Pang *et al.* (1988)
	83		
Helianthinin	–I L P G C R R T Y–	–S N G V E E T I C S M K F–	Vonder Haar *et al.* (1988)
	98		
Glutelin I	–T F P G C P E S Y–	–S N G L D E T N C T L R V–	Takaiwa *et al.* (1987b)

(continued)

TABLE XVI
(continued)

11S globulin	Acid polypeptide	Basic polypeptide	Reference
	98		
Glutelin II	–T F P G C P E T Y–	–P N G L D E T F C T M R V–	Takaiwa *et al.* (1987b)
	103	7	
Pumpkin 11S globulin	–A I P G C A E T Y–	–E N G L E E T I C T L R L–	Hayashi *et al.* (1988)
	89		
Cotton β-globulin A	–V M P G C A E T F–	–D N G L E E T F C S M R I–	Chlan *et al.* (1986)
	104		
Cotton β-globulin B	–V F P G C P E T Y–	–G N G L E E T F C S M R L–	Chlan *et al.* (1986)
	82		
Cruciferin	–R V V L C A E T F–	–V N G L E E T I C S A R C–	Simon *et al.* (1985)
	90		
Cruciferin	–V V P G C A E T F–	–D N G L E E T I C S M R T–	Rödin *et al.* (1990)

[a]The numbers above the cysteine residues are the positions from the mature N termini of the acidic and basic polypeptides. Arrow indicates the processing site between the acidic and basic polypeptides.
[b]The number includes the signal peptide region.

TABLE XVII
AMINO ACID SEQUENCES AROUND CYSTEINE RESIDUES ASSUMED TO BE INVOLVED IN INTRADISULFIDE BOND

11S globulin	Position and sequence around cysteine residue[a]		Reference
	12	45	
Glycinin $A_{1a}B_{1b}$	—QNECQIQ—	—PFQCAGV	Negoro *et al.* (1985); Utsumi *et al.* (1987b); Sims and Goldberg (1989)
	12	45	
Glycinin $A_{1b}B_2$	—QNECQIQ—	—PFQCAGV	Cho and Nielsen (1989)
	10	43	
Glycinin A_2B_{1a}	—QNECQIQ—	—PFQCAGV	Momma *et al.* (1985b); Utsumi *et al.* (1987a); Thanh *et al.* (1989)
	9	42	
Glycinin A_3B_4	—FNECQLH—	—ELQCAGV	Fukazawa *et al.* (1985)
	9	42	
Glycinin $A_5A_4B_3$	—LNECQLN—	—ELKCAGV	Momma *et al.* (1985a)
	10	43	
Pea legumin A	—QNECQLE—	—QFRCAGV	Lycett *et al.* (1984a); Rerie *et al.* (1990)

(continued)

TABLE XVII
(continued)

11S globulin	Position and sequence around cysteine residue[a]		Reference
	11	44	
Pea legumin J	—LNQCQLD—	—ELKCAGV	Gatehouse *et al.* (1988)
	10	43	
Field bean legumin A	—QNECQLE—	—QFRCASV	Schlesier *et al.* (1990)
	11	44	
Field bean legumin B_4	—LNQCRLD—	—ELRCAGV	Bäumlein *et al.* (1986)
	21	54	
Oat 12S globulin	—LRGCRFD—	—QFRCAGV	Shotwell *et al.* (1988); Schubert *et al.* (1990)
	36	69	
Arabidopsis CRA1[b]	—PNECQLD—	—QLRCSGV	Pang *et al.* (1988)
	30	63	
Arabidopsis CRB[b]	—PNECQLD—	—QLRCSGF	Pang *et al.* (1988)
	12	45	
Helianthinin	—QNQCQLQ—	—QFQCAWS	Vonder Haar *et al.*(1988)
	22	55	
Glutelin I	—PRECRFD—	—QFQCTGV	Takaiwa *et al.* (1987b)
	22	55	
Glutelin II	—PRGCRFD—	—LFQCTGV	Takaiwa *et al.* (1987b)
	27	60	
Pumpkin 11S globulin	—PRACRLE—	—EFQCAGV	Hayashi *et al.* (1988)
	13	46	
Cotton β-globulin A	—QNECQIN—	—QLRCAGV	Chlan *et al.* (1986)
	28	61	
Cotton β-globulin B	—QSQCQLQ—	—QFQCAGV	Chlan *et al.* (1986)
	7	40	
Cruciferin	—PNECQLD—	—QLRCSGV	Simon *et al.* (1985)
	14	47	
Cruciferin	—GNACNLD—	—QIRCAGV	Rödin *et al.* (1990)

[a]The numbers above the cysteine residues are the positions from the mature N termini of the acidic polypeptides.

[b]The number includes the signal peptide region.

glycinin, and sesame α-globulin within the same species (Utsumi *et al.*, 1980a, 1983; Mori *et al.*, 1982b) and also among different species (Mori *et al.*, 1979; Utsumi *et al.*, 1980a, 1983) to form artificial 11S globulins.

From the results of electron microscopy and X-ray scattering studies of glycinin (Badley *et al.*, 1975; Miles *et al.*, 1985b; I'Anson *et al.*, 1987), helianthinin (Plietz *et al.*, 1983), cruciferin (Plietz *et al.*, 1983), field bean legumin (Plietz *et al.*, 1984), pea legumin (Miles *et al.*, 1985a), and sesame α-globulin (Plietz *et al.*, 1986), some structural models have been proposed. The trigonal antiprism

model (Fig. 4) is generally accepted, although there are some discrepancies between the models and the data (Miles *et al.*, 1985b; I'Anson *et al.*, 1987) due to heterogeneity of the molecular species (Utsumi and Mori, 1980, 1981; Utsumi *et al.*, 1981) and the polymorphism of the primary structure (Utsumi *et al.*, 1987a,b). The X-ray scattering studies of the 11S globulins are reviewed by Plietz and Damaschun (1986).

Crystallizations of edestin from hemp seed and excelsin from brazil nuts (Schepman *et al.*, 1972) and cucurbitin from squash (Colman *et al.*, 1980) have been achieved. However, comprehensive X-ray analyses have not yet been undertaken, because the primary structures of edestin, excelsin, and cucurbitin have not been determined (Wright, 1987). Most of the 11S globulins have not yet

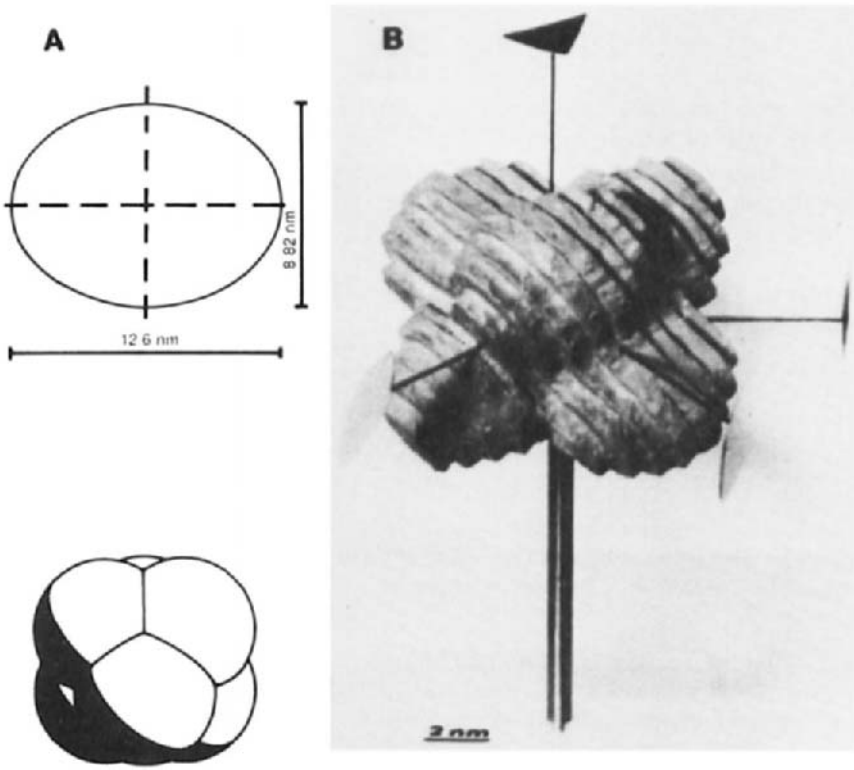


FIG. 4. Quaternary structural model of 11S globulin. (A) The model of *Vicia faba* legumin, consisting of six subunits, with the dihedral point group symmetry 32. (B) The model of the 11S globulin from sunflower seed and rapeseed, consisting of six structurally identical subunits in the arrangement of a trigonal antiprism with the dihedral point group symmetry 32. (From Plietz *et al.*, 1983, 1984, with permission of the authors and publisher.)

been crystallized. The difficulty in the crystallization of these proteins may be due to the heterogeneity of the molecular species and the polymorphism of the primary structures. However, success in the high-level expression of glycinin in *E. coli* (Kim *et al.*, 1990a) and yeast (Utsumi *et al.*, 1991) should facilitate crystallization, because such an expression system enables the production of a single molecular species.

Wright (1987, 1988) aligned the amino acid sequences of the 11S globulins to maximize their homology and suggested that they are composed of a series of alternating conserved and variable domains; that is, four conserved and five variable domains (Table XVIII). Each variable domain exists in the hydrophilic region, which suggests that they are located on the surface of the protein. Three of four conserved cysteine residues, which are assumed to be involved in inter- and intradisulfide bonds (Tables XVI and XVII), are located in conserved domain I (Table XVIII).

c. Comparison of the 7S and 11S Globulins. From the comparison of the amino acid sequences of the 7S and 11S gobulins, Gibbs *et al.* (1989) concluded that the genes of both globulins are derived from a common ancestral gene. This is consistent with the existence of homologous regions between both globulins as indicated by Wright (1987, 1988) (Tables XV and XVIII).

Argos *et al.* (1985) compared the amino acid sequences of 7S and 11S globulins

TABLE XVIII

DOMAIN STRUCTURE OF 11S GLOBULINS[a]

Domain	Aligned residue no.[b]	Characteristics
Variable I	1–25	—
Conserved I	26–113	Contains three conserved cysteine residues; homology region A (67–112); residues 73–100 homologous with residues 551–575
Variable II	114–174	Cruciferin insert present
Conserved II	175–235	Residues 184–199 homologous with residues 592–607
Variable III	236–318	—
Conserved III	319–369	Homology region B (319–344)
Variable IV	370–493	Corresponds to hypervariable region of Argos *et al.* (1985)
Conserved IV	494–655	Contains A/B processing site and single conserved cysteine residue; homology region C (533–581); homology region D (588–619); residues 551–575 homologous with residues 73–100; residues 592–607 homologous with residues 184–199
Variable V	656–723	—

[a]From Wright (1988), with permission of the author and publisher.
[b]Containing gaps.

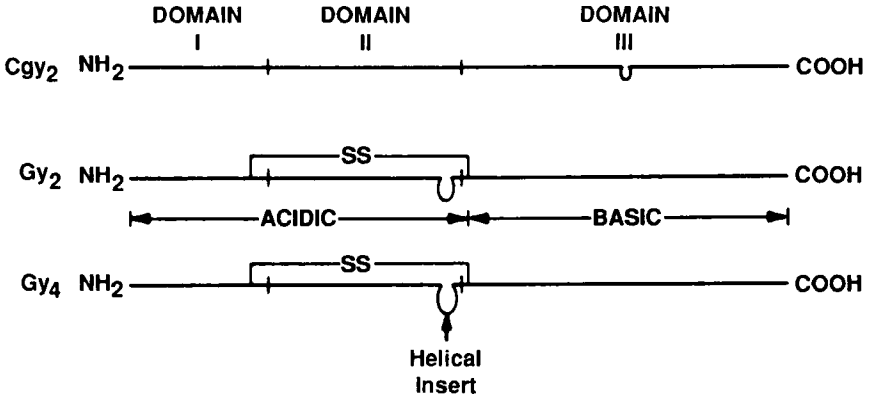


FIG. 5. The predicted domain relationships between 7S and 11S globulins. Cgy_2 corresponds to the β-conglycinin α′ subunit; Gy_2 and Gy_4 correspond to the glycinin $A_{1a}B_{1b}$ and $A_5B_4B_3$ subunits, respectively. (From Argos *et al.*, 1985, with permission of the authors and publisher.)

by considering the conformational characteristics and tendencies of the amino acids (helix, sheet, and turn secondary structural conformational preferences; residue polarity; hydrophilicity and hydrophobicity) and revealed that sequences of both globulins could be aligned and shared a number of predicted secondary structural features. They proposed the domain structure shown in Fig. 5. Domain I (N-terminal domain) is different in both globulins. Domain II (central domain) in both globulins has a common secondary structure. Domain III (C-terminal half corresponding to the basic polypeptide) is highly conserved and is largely hydrophobic. The same authors further suggested that domain III is probably buried within the molecule and plays an important role in maintaining the structure. The most conspicuous difference between the 7S and 11S globulins is the existence of a hypervariable region (helical insert) between domains II and III in the 11S globulin subunits. The hypervariable region is different in size among the 11S globulin subunits and is rich in acidic amino acids. The size difference of this region accounts for the size difference of the subunits. The 7S globulin is a trimer and the 11S globulin is a hexamer. Therefore, there is a possibility that the difference in domain I in both globulins is responsible for the quaternary structure.

III. BIOSYNTHESIS OF SEED STORAGE PROTEINS

Seed storage proteins are synthesized in the rough endoplasmic reticulum. The signal peptide is removed cotranslationally in the endoplasmic reticulum, then the storage proteins are deposited directly into this compartment or are

transported and deposited into the protein bodies. The legumes generally accumulate the storage proteins in the cotyledons, whereas the cereals accumulate them in scutellar and/or endosperm cells.

A. STORAGE PROTEINS OF CEREALS

Maize zeins and sorghum prolamins are deposited directly into the endoplasmic reticulum to form the protein bodies (Larkins and Hurkman, 1978; J. R. N. Taylor *et al.*, 1985). In rice, prolamins are also deposited directly into the endoplasmic reticulum, but glutelins are transported via the golgi apparatus and are deposited into the protein bodies like the 11S globulins (Krishnan *et al.*, 1986). This observation is consistent with the fact that rice seeds have two types of protein bodies, PB-I and PB-II; PB-I contains prolamins and has a concentric ring structure, whereas PB-II contains glutelin (Yamagata *et al.*, 1982; Yamagata and Tanaka, 1986). Masumura *et al.* (1990) observed a difference in the amino acid sequences of the signal peptides of rice prolamins and glutelins and suggested that the signal peptides may play an essential role in the process of polypeptide accumulation in the protein bodies. In wheat, gliadins are transported via the golgi apparatus and are deposited into the protein bodies (Kim *et al.*, 1988). This is also the case with γ-secalin of rye (Krishnan *et al.*, 1990). In oats, avenin seems to be deposited directly into the rough endoplasmic reticulum and then transported into the vacuole to form the protein bodies (Lending *et al.*, 1989). However, the oat 11S globulin appears to aggregate mainly in the vacuole in the same way as that for avenin, but the two proteins are spatially separated (Lending *et al.*, 1989).

B. STORAGE PROTEINS OF LEGUMES

Some of the constituent subunits (proform or mature form) of the 7S globulins synthesized and translocated in the lumen of the endoplasmic reticulum are cotranslationally N-glycosylated and assemble into trimers (Chrispeels *et al.*, 1982a,b; Shotwell and Larkins, 1989). The trimers are transported into the vacuole via the golgi apparatus to form the protein bodies. During this transportation, pea vicilin processing and oligosaccharide chain modifications occur (Chrispeels *et al.*, 1982a; Shotwell and Larkins, 1989). Processing of the proform of β-conglycinin α′ and α may also occur. The N-glycosylation is, however, not necessary for vicilin to be synthesized, transported, or assembled into trimers (Badenoch-Jones *et al.*, 1981).

The constituent subunits (proform) of the 11S globulins, which are synthesized and translocated in the lumen of the endoplasmic reticulum, assemble into trimers (Barton *et al.*, 1982; Chrispeels *et al.*, 1982b) and the disulfide bonds between their acidic and basic polypeptides are formed (Hara-Nishimura, 1987). Monma

et al. (1988a,b) observed a difference in the subcellular localization in β-conglycinin and glycinin in the developing soybean cotyledons and isolated glycinin-rich organelles. These results indicate that the translocation of the 11S globulin into the protein bodies is different from that of the 7S globulin. The processing of proglobulin to form the acidic and basic polypeptides and their assembly into hexamers occur in the protein bodies (Chrispeels *et al.*, 1982b). The presence of a processing enzyme that cleaves the junction of the acidic and basic polypeptides in the vacuole or the protein bodies has been established in pumpkin seeds (Hara-Nishimura and Nishimura, 1987), castor bean (Fukasawa *et al.*, 1988), and soybean (M. P. Scott *et al.*, personal communication). The processing enzyme of pumpkin seeds is a thiol enzyme with an optimum pH of 5.0 (Hara-Nishimura and Nishimura, 1987). In soybean seeds, the processing enzyme is a glycosylated cysteine protease with a molecular mass of 40 kDa and the cleavage of the junction is sequence specific (M. P. Scott *et al.*, personal communication). Saalbach *et al.* (1991) demonstrated that long amino-terminal and short carboxy-terminal domains of the legumin propolypeptide of field bean act as vacuolar targeting signals.

The 7S and 11S globulins are differentially regulated, with the 7S globulins accumulating faster than the 11S globulins (Meinke *et al.*, 1981; Gatehouse *et al.*, 1986). The constituent subunits of β-conglycinin and glycinin are also differentially regulated. In the case of β-conglycinin, α and α′ subunits are synthesized faster than the β subunit (Meinke *et al.*, 1981; Naito *et al.*, 1988), whereas the A_3B_4 subunit is the last among the glycinin subunits to be synthesized (Meinke *et al.*, 1981). On the other hand, the content of the 7S and 11S globulins in the seeds is affected by environmental factors (Wright, 1987). An increased β-conglycinin level, with a preferential increase of the β subunit, and a decreased glycinin level were observed in the seeds of soybeans grown under sulfur-deficient conditions (Gayler and Sykes, 1985). In addition, supplemental methionine and methionine analogs inhibit production of the β subunit of β-conglycinin in cultured soybean cotyledons (Creason *et al.*, 1985; Holowach *et al.*, 1986). A similar effect was observed with pea seeds (Chandler *et al.*, 1983; Beach *et al.*, 1985; Evans *et al.*, 1985). These facts suggest that the regulation mechanism of the expression of the 7S and 11S globulin genes and that of the constituent subunit genes differ.

IV. FUNCTIONAL PROPERTIES OF SEED STORAGE PROTEINS

Wheat flours are used for making bread and noodles, and soybean seeds are used for making a variety of oriental traditional foods, including tofu, kooridofu, yuba, and many others. Some seed proteins, including wheat and soybean proteins, can be utilized as food ingredients and for the manufacture of fabricated

and processed foods (Kinsella *et al.*, 1985). Whether seed proteins are able to be utilized for such foods is determined by their functional properties. In other words, the functional properties of seed proteins determine their food applications in specific food systems, and their acceptability (Kinsella *et al.*, 1985). Functional properties of importance in food applications are listed in Tables XIX and XX. These properties vary with protein source, protein concentration, protein fraction, prior treatment, and environmental conditions (pH, temperature, ionic strength, etc.) (Kinsella *et al.*, 1985).

V. RELATIONSHIPS BETWEEN STRUCTURE AND FUNCTIONAL PROPERTIES OF SEED STORAGE PROTEINS

A. GELATION

The capacity of seed proteins to form gel is one of the most important functional properties with respect to texture formation. The ability of gels to act as a matrix for holding water, lipids, sugars, flavors, and other ingredients is useful in food applications and in new product development (Kinsella, 1979).

1. Soybean Glycinin

For glycinin, the lowest protein concentration to form heat-induced gels is 2.5% at 100°C, pH 7.6, and ionic strength $\mu = 0.5$ (Utsumi *et al.*, 1982). Gel hardness increases with increasing protein concentration. At lower protein concentrations, glycinin solutions become turbid (Mori *et al.*, 1982a; Utsumi *et al.*, 1982). The overall scheme of the thermal change in glycinin is illustrated in Fig. 6 (Mori *et al.*, 1982a, 1986; Nakamura *et al.*, 1984b). A brief description of the proposed scheme is as follows: (1) Glycinin molecules unfold moderately on heating but still remain globular, associating as soluble aggregates composed of "strings of beads" with molecular masses of 8000 kDa or as aggregates with molecular masses of 1800 and 4000 kDa. The SH/S—S exchange reaction is necessary to form branched strands. (2) The 8000-kDa soluble aggregates associate with each other to form network structures by SH/S—S exchange reactions via soluble macroaggregates (>80,000 kDa). At low protein concentrations (step 2*, Fig. 6), the soluble aggregates dissociate into acidic and basic polypeptides. The basic polypeptides form insoluble aggregates due to their hydrophobic nature (Mori *et al.*, 1982a). Step 2* proceeds even at high protein concentrations, although to a much lesser extent at increasing concentrations. (3) Subsequent heating stabilizes the network structure to form a matured gel. A temperature of 100°C is required for step 1 to proceed at pH 7.6 and $\mu = 0.5$, but this is not the case for steps 2 and 3 (Nakamura *et al.*, 1985b).

TABLE XIX

FUNCTIONAL PROPERTIES OF SEED PROTEINS OF IMPORTANCE IN FOOD APPLICATIONS[a]

General property	Specific functional attribute
Organoleptic	Color, flavor, odor
Kinesthetic	Texture, mouthfeel, smoothness, grittiness, turbidity, chewiness
Hydration	Wettability, water absorption, water-holding capacity, swelling, solubility, thickening, gelling, syneresis
Surface	Emulsification, foaming (aeration, whipping), protein–lipid film formation, lipid binding, flavor binding
Structural and rheological	Viscosity, elasticity, adhesiveness, cohesiveness, stickiness, dough formation, aggregation, gelation, network formation, extrudability, texturizability, fiber formation
Other	Compatibility with other food components, enzymatic activity, antioxidant properties

[a]From Kinsella (1979), with permission of the author and publisher.

TABLE XX

TYPICAL PROPERTIES CONFERRED BY SEED PROTEINS TO FOOD SYSTEMS[a]

Functional property	Mode of action	Food system
Solubility	Protein solvation	Beverages
Water absorption and binding	Hydrogen bonding of water, entrapment of water (no drip)	Meats, sausages, breads, cakes
Viscosity	Thickening, water binding	Soups, gravies
Gelation	Protein matrix formation and setting	Meats, curds, cheese
Cohesion–adhesion	Protein action as adhesive material	Meats, sausages, baked goods, cheeses, pasta products
Elasticity	Hydrophobic bonding and disulfide links in gluten; disulfide links in gels	Meats, bakery products
Emulsification	Formation and stabilization of fat emulsions	Sausages, bologna, soup, cakes
Fat absorption	Binding of free fat	Meats, sausages, doughnuts
Flavor binding	Adsorption, entrapment, release	Simulated meats, bakery goods
Foaming	Formation of stable films to entrap gas	Whipped toppings, chiffon desserts, angel food cakes

[a]From Kinsella (1979), with permission of the author and publisher.

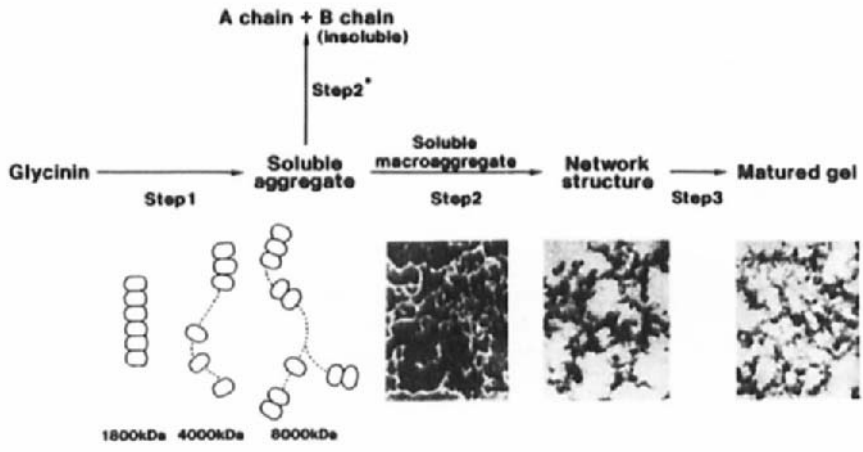


FIG. 6. Process of heat-induced gelation of glycinin. Soluble aggregates are represented as strings of beads (in an abbreviated form, with dashed lines). Each component of the string represents a moderately unfolded glycinin molecule. Photographs of a soluble macroaggregate, a network structure, and a matured gel are shown.

Hermansson (1985) also proposed a "string of beads" model for glycinin gelation by a process similar to that described above, with the exception of the mechanism of trigger of association. Hermansson suggested that the glycinin molecules dissociate into constituent subunits and reassemble into an 11S form with the formation of gels.

The subunit composition of glycinin varies among cultivars (Mori *et al.*, 1981), and it is possible to create artificial glycinin molecules with unique subunit compositions using isolated subunits or the acidic and basic polypeptides (Utsumi *et al.*, 1980a; Mori *et al.*, 1982b; Nakamura *et al.*, 1985a). Comparison of the gelation processes and properties of gels among various native and artificial glycinins with different subunit compositions reveals the relationships between structural characteristics and gelation.

The heating time required for gel formation at 100°C, pH 7.6, and $\mu = 0.5$ was compared among glycinins with different subunit compositions from five soybean cultivars—Shiro Tsuru-no-ko, York, Hill, Matsuura, and Raiden (Nakamura *et al.*, 1984a). Glycinins having $A_5A_4B_3$ subunits (Shiro Tsuru-no-ko, York, and Hill) formed gels within half the time required for glycinins having no $A_5A_4B_3$ subunit (Matsuura and Raiden). Thus the $A_5A_4B_3$ subunit may correlate with ease of gelation. Since the A_4 polypeptide associates with disulfide-bonded A_5–B_3 through noncovalent interactions, the A_4 polypeptide has a tendency to be liberated when the glycinin molecule is heated. The liberation of A_4 polypeptide could result in a conformational change of the glycinin molecule, stimulating the formation of soluble aggregates and subsequent polymerization,

resulting in rapid gel formation (Nakamura *et al.*, 1984a). In other words, the heat instability of the constituent subunits may be related to the heat-induced gel-forming ability.

The lowest protein concentration for glycinin gelation was 2.5% in all the cultivars, and gel hardness increased with increasing protein concentrations (Nakamura *et al.*, 1984a). At higher protein concentrations (>7.5%), the hardness of the gels was directly proportional to the percentage of A_3B_4 subunit (Nakamura *et al.*, 1984a). This gel hardness relationship was also observed for artificial glycinins with different subunit compositions reconstructed from isolated subunits (Nakamura *et al.*, 1985a) and polypeptides (Mori *et al.*, 1982b). The artificial glycinin composed of the A_3B_4 subunit gave a gel in which the length and extent of branching of the constituent strands were, respectively, shorter and much greater in number, as compared to those in the native glycinin gel (Nakamura *et al.*, 1985a). On the other hand, the gel from artificial glycinin, composed of the A_2B_{1a} subunit, the hardness of which was lower than that of the gels from artificial glycinins composed of the other subunits, exhibited a rough network structure, wherein the strand length was longer and the extent of branching was smaller. Therefore, glycinin gel hardness may be determined by the strand length and extent of branching in the network structure of the gel, i.e., the elaborateness of the gel network, to which the A_3B_4 subunit largely contributes. The molecular size of the A_3B_4 subunit is bigger than those of the $A_{1a}B_{1b}$, $A_{1b}B_2$, and A_2B_{1a} subunits. Moreover, the number of free cysteine residues and the amino acid sequences around the free cysteine residue near the C terminus of the acidic polypeptide are different among the constituent subunits (Table XXI). These factors may be related to gel hardness.

The turbidity of artificial glycinin gels due to the $A_{1a}B_{1b}$, A_2B_{1a}, or A_3B_4 subunits can be ranked in the following order: $A_2B_{1a} \gg$ native glycinin $>$ $A_{1a}B_{1b} > A_3B_4$ (Nakamura *et al.*, 1985a). It was suggested that the sulfhydryl group concentration could be responsible for the turbidity (Nakamura *et al.*, 1984a, 1985a). However, the concentration of sulfhydryl groups is the same in the $A_{1a}B_{1b}$ and A_2B_{1a} subunits, and the concentration of A_3B_4 is less than that of $A_{1a}B_{1b}$ and A_2B_{1a}. Therefore, the chemical properties around the cysteine residues may be related to the turbidity.

2. *Vicia faba Legumin*

Vicia faba legumin can form a heat-induced gel, but the lowest protein concentration required for gelation at 100°C, pH 7.6, and $\mu = 0.5$ is much higher (10%) than that of glycinin (Utsumi *et al.*, 1983). The constituent acidic polypeptides (ASI, ASII, and ASIII) were isolated by DEAE–Sephadex column chromatography in the presence of 6 *M* urea and 0.2 *M* 2-mercaptoethanol, and then artificial legumins were reconstituted from each acidic polypeptide and the

TABLE XXI

AMINO ACID SEQUENCES AROUND FREE CYSTEINE RESIDUES

Subunit	Position and sequence around cysteine residue[a]		Reference
	Acidic polypeptide	Basic polypeptide	
	53 271 278	86	
$A_{1a}B_{1b}$	GVALSRCTLNRNA–EDELPQCKGKDKHCQRPRGS–	–LIQVVNCNGERVG–	Negoro *et al.* (1985); Utsumi *et al.* (1987b); Sims and Goldberg (1989)
	51 266 273	86	
A_2B_{1a}	GVALSRCTLNRNA–EEEQPQCVETDKGCQRQSKR–	–LVQVVDCNGERVF–	Momma *et al.* (1985b); Utsumi *et al.* (1987a); Thanh *et al.* (1989)
	53 264 271	86	
$A_{1b}B_2$	GVALSRCTLNRNA–EEEKPDCDEKDKHCQSQSRN–	–LVQVVNCNGERVF–	Cho and Nielsen (1989)
	316	86	
A_3B_4	–EPRGRGCQTRNGV–	–RVRVVNCQGNAVF–	Fukazawa *et al.* (1985)
	350	86	
$A_5A_4B_3$	–EPRERGCETRNGV–	–KVRVVNCQGNAVF–	Momma *et al.* (1985a)

[a]The numbers above the cysteine residues are the positions relative to the N terminus of the acidic and basic polypeptides.

mixture of the basic polypeptides (Utsumi and Mori, 1983). Comparisons of the properties of the gel from each reconstituted legumin demonstrated that ASII plays an important role in gelation and in increasing gel hardness, and that ASIII contributes to the gel transparency (Utsumi *et al.*, 1983). The amino acid sequences of some constituent subunits of legumin were determined (Table XI). However, the nature of the subunit that contains ASI, ASII, or ASIII is unknown. Therefore, the difference in the contribution of each acidic polypeptide to the gelation and gel properties is not explained by the amino acid sequence.

B. EMULSIFICATION

The ability of protein to form and stabilize emulsions is necessary for the production of mayonnaise, salad dressings, sausages, whiteners, and many other products, and as such it is one of the most important functional properties for food systems (Kinsella *et al.*, 1985). Proteins having an amphipathic structure with hydrophilic and hydrophobic regions that are polarized in the molecules can exhibit emulsifying ability. In oil–water systems, proteins migrate to and adsorb at the interface, and then unfold to a limited extent, depending on their structure, and interassociate there (Kinsella *et al.*, 1985). The structural characteristics and solubility in the aqueous phase are closely related to the emulsifying ability of proteins (Kinsella *et al.*, 1985).

Kato and Nakai (1980) demonstrated significant correlations between surface hydrophobicity and surface properties of proteins. This was confirmed by linear correlation between emulsion stability and the surface hydrophobicity of heated glycinins at different ionic strengths (Matsudomi *et al.*, 1985). In addition, chemical attachment of fatty acyl residues to glycinin increased its surfactant properties (Utsumi and Kito, 1991). Acylated glycinin exhibited better emulsification ability than did the native glycinin (Nishimura *et al.*, 1989). These results indicate that amphiphilicity is an important factor in evaluating the emulsifying properties of glycinin.

C. BREADMAKING QUALITY

Mixing wheat flour with water causes formation of a viscoelastic dough, resulting from the formation of a gluten matrix. Gluten plays important roles in the breadmaking quality of wheat flour. Of the main constituents of gluten, gliadins contribute to extensibility, whereas glutenins contribute to a toughness and elasticity that prevents the dough from being overextended and from collapsing (Payne, 1983).

Bread wheat flours have a protein composition that is suitable for breadmaking, but their protein composition varies among wheat cultivars. Based on comparisons of the constituent proteins of various wheat cultivars with

different breadmaking qualities, it was suggested that HMW glutenin aggregates contribute to the desirability of a cultivar for use in breadmaking (Huebner and Wall, 1976). Payne *et al.* (1979, 1981) demonstrated a significant correlation between the breadmaking quality (the volumes of the loaves) and certain HMW glutenin subunits by means of SDS–PAGE and backcrossing. These findings were confirmed later by many other workers (Branlard and Dardevet, 1985; Moonen and Zeven, 1985; Lagudah *et al.*, 1987, 1988; Lawrence *et al.*, 1987, 1988; Payne *et al.*, 1987a,b, 1988a,b).

Tatham *et al.* (1985) studied conformations of wheat gluten proteins by means of a combination of CD and computer prediction of their secondary structure based on their amino acid sequences. By comparing wheat gluten proteins with the β-spiral model proposed by Venkatachalam and Urry (1981) for elasticity of the mammalian elastomeric protein (elastin), Tatham *et al.* (1985) proposed the model to explain how HMW glutenins contribute to dough elasticity. The β spirals are helical structures consisting of repetitive β turns with 13.5 residues per turn. As described in the Section II, the central repetitive domains (75–85% of the molecule) of HMW glutenin subunits are predicted to be composed of regularly repeated β turns, resulting in the formation of β spirals, as in elastin (Tatham *et al.*, 1985). If this is the case, the repetitive domain would be 44–45 × 1.8 nm (Shewry and Tatham, 1990). The dimension calculated from the intrinsic viscosity is from 49 × 1.8 nm to 62 × 1.5 nm. Thus, the theoretical value agrees well with the calculated value. Scanning tunneling microscopy provided direct evidence for the formation of such a spiral structure (Miles *et al.*, 1991). HMW glutenin subunit 10 is associated with good breadmaking quality, and subunit 12 is associated with poor breadmaking quality (Payne *et al.*, 1981; Lagudah *et al.*, 1987). The secondary structure predicted from their amino acid sequences was compared (Flavell *et al.*, 1989). The comparison indicated that subunit 10 has a more regular pattern of repetitive β turns than does subunit 12. In other words, subunit 10 has a stronger β spiral with better elastic properties (Flavell *et al.*, 1989). The difference in the structures of subunits 10 and 12 was directly demonstrated by SDS–PAGE (Goldsbrough *et al.*, 1989). Multicysteine and one-cysteine residues are present in the unique N-terminal and C-terminal domains, respectively. This would permit the formation of linear (head to tail) polymers with some branching, cross-linking, and interaction with LMW glutenin subunits at the N-terminal domain (Tatham *et al.*, 1985; Shewry and Tatham, 1990). These observations strongly suggest that the HMW glutenin structure (β spiral) proposed by Tatham *et al.* (1985) could contribute to elasticity of the dough.

Based on amino acid sequences, C-hordein was also suggested to form β-spiral structures (Shewry and Tatham, 1990). The ω-gliadin exhibits structural characteristics similar to that of C-hordein. Therefore, ω-gliadin is presumed to form β-spiral structures, but has no cysteine residue and as such it cannot form

an elastic polymer (Tatham *et al.*, 1985). The N-terminal repetitive domain (~40% of the molecule) of γ-gliadin may form β spirals and the C-terminal domain (~60% of the molecule) is probably compact (Shewry and Tatham, 1990). α/β-Gliadin and LMW glutenin may not form β spirals because of the greater irregularity of the β turns (Shewry and Tatham, 1990). On the other hand, a contribution of LMW glutenins to dough resistance and extensibility has been suggested by Gupta *et al.* (1989). If this is the case, the mechanism of the contribution must be different from that of HMW glutenins.

D. BISCUITMAKING QUALITY

A dough from wheat flour to be used for biscuitmaking must have a good extensibility and poor elasticity (Payne, 1983). The extensibility of a dough is contributed by gliadins, although it has not yet been elucidated which gliadins contribute. It has been pointed out that hard wheat cultivars that are suitable for breadmaking are not good for biscuitmaking (Payne *et al.*, 1987a).

E. PASTA COOKING QUALITY

Durum wheat is used to make pasta, the cooking quality of which depends mainly on the durum wheat gluten. The criteria for cooking quality, i.e., viscoelasticity and the surface condition of a cooked pasta, have been well documented (Feillet *et al.*, 1989). A significant relationship was demonstrated between γ-gliadin components and viscoelasticity (Kosmolak *et al.*, 1980; Feillet *et al.*, 1989). Glutens from durum wheat cultivars with γ-gliadin 45 exhibit better firmness and viscoelasticity than those from other cultivars with γ-gliadin 42. Gluten firmness and viscoelasticity are properties contributed by glutenin rather than by gliadin (Payne *et al.*, 1984; Pogna *et al.*, 1988). The ω-gliadins 33, 35, and 38 and LMW-1 glutenin subunits are genetically linked to γ-gliadin 42, and ω-gliadin 35 and LMW-2 glutenin subunits are linked to γ-gliadin 45 (Payne *et al.*, 1984; Shewry *et al.*, 1986). Therefore, it is conceivable that the type of γ-gliadin is a genetic marker for pasta firmness and elasticity (Feillet *et al.*, 1989). Pogna *et al.* (1988) found a wheat variety (Berillo) that contains γ-gliadin 42, ω-gliadin 35, and LMW-2 glutenin subunits; this variety has a high degree of gluten elastic recovery and firmness, corresponding to the same characteristics seen in γ-gliadin 45-type cultivars. This suggests that LMW glutenin subunits are the functional proteins of gluten viscoelasticity and firmness (Pogna *et al.*, 1988; Feillet *et al.*, 1989). This was confirmed by studies comparing various cultivars: (1) Type 45 cultivar contained more LMW glutenin subunits than did type 42 cultivar; (2) good correlations were found between pasta cooking quality and the concentrations of 0.1 *M* acetic acid-insoluble proteins (Sgrulletta and Stefanis, 1989), and also between pasta firmness and elastic recovery and

concentrations of LMW glutenin aggregates (Feillet *et al.*, 1989); and (3) HMW glutenin subunits were found to be poor indicators of pasta viscoelastic properties (Du Cros, 1987). Analysis of the progenies of crosses between Berillo and four durum wheat cultivars confirmed that the type of γ-gliadin is only a genetic marker (Pogna *et al.*, 1990). Moreover, the same authors indicated that LMW-2 glutenin subunits are the most important proteins for gluten quality, although some HMW glutenin subunits correlate with gluten quality, and their effect is additive to that of LMW glutenin subunits.

Pasta is exposed to heat treatment during drying operations and cooking. Analyses of the behavior of pasta proteins under well-defined hydrothermic conditions indicated that LMW glutenin subunits are aggregated by disulfide bond formation. This property of LMW glutenin subunits may be important in determining pasta cooking quality (Feillet *et al.*, 1989).

Alary and Kobrehel (1987) observed a highly significant correlation between the concentrations of sulfhydryl and disulfide groups in glutenin and the surface condition of cooked pasta in various durum cultivars. Kobrehel *et al.* (1988) suggested that durum wheat sulfur-rich glutenin (DSG-1, 14.4 kDa; DSG-2, 17.7 kDa) contributes to the surface condition of cooked pasta.

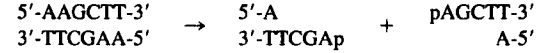
VI. GENE CLONING

Protein engineering to improve the quality of food proteins and elucidate the relationships between the structure and the functional properties of food proteins requires the isolation of genomic DNAs or cDNAs encoding the target proteins. A brief procedure of genomic DNA and cDNA cloning is presented in Fig. 7. The recent development of techniques, enzymes, and vectors related to cloning make the procedure outlined in Fig. 7 routine. The enzymes, vectors, and techniques will be described below in detail.

A. ENZYMES INVOLVED IN GENE CLONING AND MANIPULATION

1. Restriction Enzymes

Restriction enzymes recognize, bind to, and cleave specific sequences of DNA. Most restriction enzymes recognize sequences four, five, or six nucleotides in length. *Hin*dIII recognizes the following sequence and cleaves it to produce fragments with protruding termini (cohesive ends):



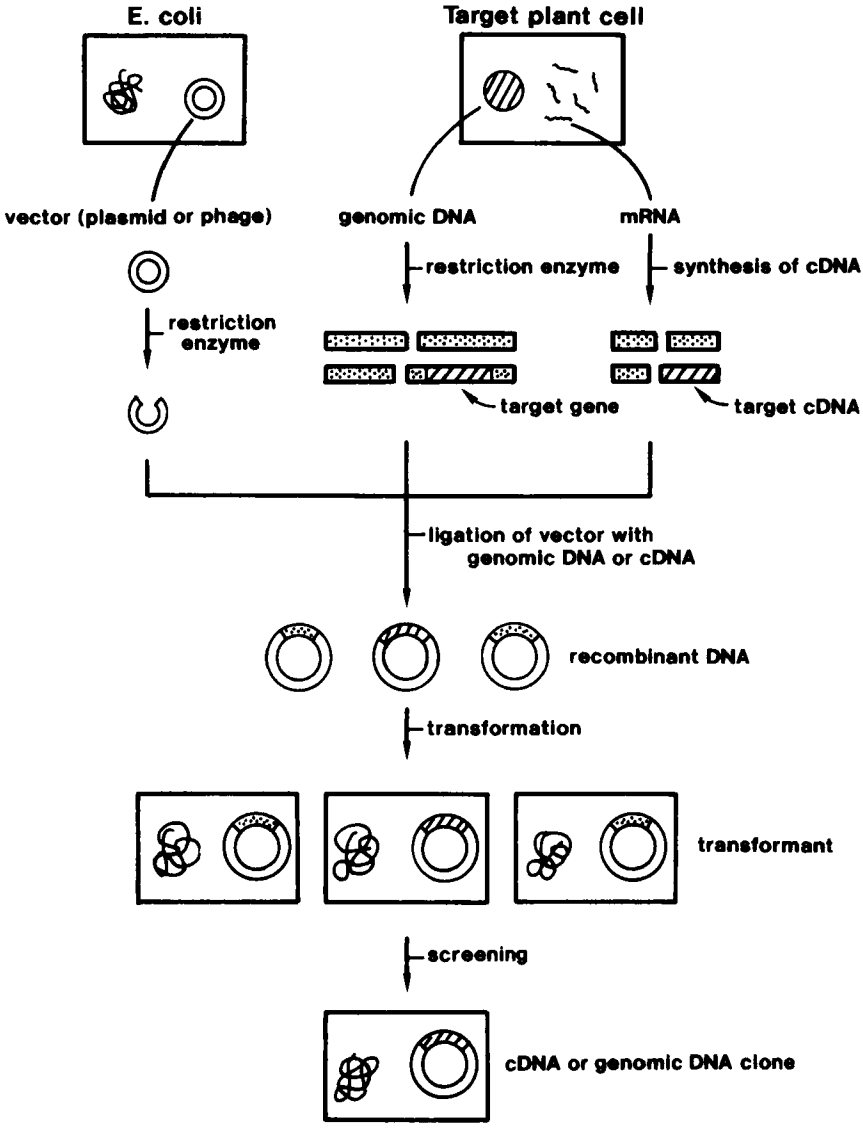


FIG. 7. Procedures for cloning cDNA and genomic DNA. See text for details.

*Sma*I recognizes the following sequence and cleaves it to produce fragments with blunt ends:

$$\begin{array}{l} 5'\text{-CCCGGG-}3' \\ 3'\text{-GGGCCC-}5' \end{array} \rightarrow \begin{array}{l} 5'\text{-CCC} \\ 3'\text{-GGGp} \end{array} + \begin{array}{r} \text{pGGG-}3' \\ \text{CCC-}5' \end{array}$$

Many kinds (more than 100) of restriction enzymes with different recognition sequences and cleavage sites are now available from many manufacturers.

2. *Other DNA/RNA-Modifying Enzymes*

DNA/RNA-modifying enzymes, which are useful and necessary for gene manipulation, are commercially available. Their properties and uses are listed in Table XXII.

TABLE XXII

PROPERTIES AND USES OF ENZYMES USED IN GENE MANIPULATION

Enzyme	Property	Use
Methylase	Methylates the recognition sites of restriction enzymes	Prevents cleavage by restriction enzymes
DNA polymerase	Synthesizes DNA form 5′ to 3′ in the presence of template and primer	
E. coli DNA polymerase I	Contains exonuclease activity for single-stranded DNA 3′ → 5′ and double-stranded DNA 5′ → 3′	Nick translation, second-strand synthesis of cDNA
Klenow fragment of *E. coli* DNA polymerase I	Contains exonuclease activity for single-stranded DNA 3′ → 5′	M13 dideoxy sequencing, blunting of protruding end, second-strand synthesis of oligonucleotide-directed mutagenesis
T4	Contains exonuclease activity for single-stranded DNA 3′ → 5′ and double-stranded DNA 5′ → 3′ (very strong)	3′ end labeling, blunting of protruding end
Taq	Heat stable	Polymerase chain reaction
Reverse transcriptase	Requires template (DNA or RNA) and primer	First-strand synthesis of cDNA
Terminal deoxynucleotidyl transferase	Requires primer but not template	Homopolymer tailing, 3′ end labeling
DNA ligase	Joins 3′-OH end with 5′-P end	Ligation of DNA fragments
T4	Both cohesive and blunt ends	
E. coli	Only cohesive end	

(continued)

TABLE XXII
CONTINUED

Enzyme	Property	Use
T4 polynucleotide kinase	Phosphorylates 5′-OH end	5′ end labeling, phosphorylation of synthesized DNA
Alkaline phosphatase	Removes 5′-phosphate	Prevents self-ligation of DNA, pretreatment for 5′ end labeling
E. coli (BAP)	Very stable	
Calf intenstine	More unstable than BAP	
Nuclease	Cleaves DNA or RNA	
S1	Endonuclease specific to single-stranded DNA and RNA	Blunting of protruding end, S1 mapping
Mung bean	Endonuclease specific to single-stranded DNA	Blunting of protruding end, S1 mapping
Bal31	Endonuclease specific to single-stranded DNA and 3′-exonuclease	Restricted digestion of DNA strand from the termini
Ribouclease H	Endoribonuclease specific to RNA strand of DNA–RNA hybrid	Second-strand synthesis of cDNA
RNA polymerase (SP6, T7)	Synthesizes RNA from 5′ to 3′ in the presence of double-stranded DNA containing SP6 or T7 promoter	Synthesis of single-stranded RNA for hybridization probe, *in vitro* translation

B. CLONING VECTORS

In order to clone genomic DNA fragments and cDNA encoding a target protein, it is necessary to ligate them with a cloning vector. Vectors employed for cloning should (1) have proper restriction enzyme site(s) for constructing recombinant DNA, (2) be able to transform host cells, and (3) be able to replicate in host cells; the transformants should be specifically and easily detectable. Cloning vectors are classified into three groups—plasmids, cosmids, and bacteriophage λ.

1. Plasmid Vectors

Plasmids are double-stranded closed circular DNA molecules carrying a replicon, which is necessary for replication (Sambrook *et al.*, 1989). Plasmids can

transform *Escherichia coli* cells that have been treated with reagents such as $CaCl_2$ (competent cells). The transformation efficiency of larger plasmids (>15 kb) is very low. In other words, the longer the length of DNA, the lower the efficiency of cloning. Most plasmid vectors contain genes encoding enzymes that degrade antibiotics, e.g., ampicillin, tetracycline, and kanamycin. These properties are used to detect transformants. Recently developed plasmid vectors contain polycloning sites where plural unique restriction sites are arranged. Plasmid vectors of the pUC and pGEM series are useful and commercially available.

2. *Phage Vectors*

The genomic DNA of bacteriophage λ is a linear double-stranded DNA molecule with single-stranded complementary termini 12 nucleotides long (cohesive termini) (Sambrook *et al.*, 1989). On infection with phage λ, phage DNA enters the host cells and the cohesive termini associate to form a closed circular DNA. After this stage, there are two pathways of growth—lytic and lysogenic. Lytic growth causes the formation of plaques. This property is used for selection of recombinant DNA. The λ DNA is 49 kb long, and the central one-third portion is not necessary for lytic growth. The essential left and right arms are ~20 and ~10 kb long. The DNA that forms the phage particle is 38–52 kb long, corresponding to 78–105% of the wild-type λ DNA. Many phage vectors derived from phage λ have been developed for gene cloning (Table XXIII). Since the

TABLE XXIII

CHARACTERISTICS OF λ PHAGE VECTORS FOR GENE CLONING

Vector	Size (kb)			Cloning site
	Left	Right	Insert	
Charon 4A	11.0	19.5	7–20	*Eco*RI
	24.8	20.5	0–6	*Xba*I
Charon 32	19.5	11.9	8–19	*Eco*RI
	20.0	13.3	5–18	*Hin*dIII
	27.0	16.8	0–10	*Sac*I
Charon 34/Charon 35	19.5	10.6	9–20	Polycloning sites (six recognition sites)
	19.5	9.6	10–21	
Charon 40	19.2	9.6	9.2–24.2	Polycloning sites (sixteen recognition sites)
EMBL 3/EMBL 4	19.9	8.8	7–20	*Sal*I, *Bam*HI, *Eco*RI
λ DASH/λ FIX	20.0	9.0	9–22	Polycloning sites (seven recognition sites)
λgt10	32.7	10.6	0–6	*Eco*RI
λgt11	19.5	24.2	0–7.2	*Eco*RI
λ ZAP	21.8	18.1	0–10	Polycloning sites (six recognition sites)

efficiency of infection by phage λ is constantly high, regardless of the insertion size, phage λ vectors are preferred for constructing gene libraries. It is important to choose vectors according to the type of cloning being attempted: cDNA cloning or genomic DNA cloning. The phage vectors, of which the possible insertion size is 0–10 kb, are suitable for cDNA cloning, whereas 5–20 kb are required for genomic DNA cloning.

3. Cosmid Vectors

Cosmid vectors have a plasmid replication origin (usually ColEl) and *cos* sequences, which are required for packaging the DNA into phage particles (Sambrook *et al.*, 1989); as such, cosmid vectors share the advantages of both plasmid and phage vectors. Therefore, it is possible to clone large segments (~45 kb) of genomic DNA, resulting in the reduction of the size of the genomic DNA library to be screened.

C. cDNA CLONING

As described in Section II, genes encoding 7S and 11S globulins and glutelins have introns. In this case, cDNA is required for protein engineering employing microorganisms. Moreover, cDNA is useful as a screening probe for genomic DNA cloning. In order to synthesize and clone cDNAs containing the whole coding sequences for the proteins, the factors affecting cDNA cloning listed in Table XXIV should be considered. Since the proportion of mRNA for seed storage proteins is usually high, cloning cDNA encoding these proteins is not difficult.

1. Isolation of mRNA

As described in Section II, seed storage proteins are synthesized during seed maturation. Mature seeds contain little intact mRNA for storage proteins, therefore, these mRNAs should be isolated from immature seeds. If the timing of the target protein synthesis is not known, it is desirable to prepare the mRNA from immature seeds with different maturation periods.

TABLE XXIV

FACTORS AFFECTING cDNA CLONING

Integrity of mRNA
Purity of enzymes employed for cDNA synthesis
Method for cDNA synthesis
Tranformation efficiency

RNase, which degrades RNA, is very stable and is thus a common contaminant from glassware, reagent solutions, and workers' hands. Therefore, when handling RNA, it is necessary to wear disposable gloves, to bake glassware, and to autoclave or treat solutions with diethyl pyrocarbonate.

There are two sources of mRNA—polyribosomes and total RNA. Since seeds also contain RNase, it is desirable to use strong RNase inhibitors (RNasin or vanadyl–ribonucleoside complexes) for the extraction of polyribosomes and to extract directly the total RNA from seeds using strong denaturants (guanidinium thiocyanate, guanidine HCl, or SDS–phenol) (Sambrook *et al.*, 1989). The poly(A) tail at the 3′ end of mRNA interacts specifically with oligo(dT) and poly(U). mRNA or poly(A) RNA can be separated from ribosomal and transfer RNAs by oligo(dT)–cellulose or poly(U)–Sepharose column chromatography. mRNA can be separated from polyribosomes to a high degree of purity. Polyribosomes synthesizing the polypeptides of target proteins can be concentrated by antibodies. In performing direct extractions of total RNA with strong denaturants, each investigator should first examine which denaturant is best for the specific target mRNA, because the concentrations of polysaccharides, lipids, and proteins are different among species. For example, the recovery of total RNA from soybean immature seeds with guanidinium thiocyanate is very low, although this denaturant is widely used for mammalian cells. Methods using SDS–phenol (Fukazawa *et al.*, 1985) and vanadyl–ribonucleoside complexes (Utsumi *et al.*, 1987b) are more suitable for the isolation of mRNA from soybeans. Since the size of mRNA is predicted from the size of the target protein it encodes, enrichment of the mRNA of interest is achieved by size fractionation methods, for example, sucrose density gradient centrifugation. Before synthesis of cDNA, the integrity of the mRNA should be examined using an *in vitro* translation system (rabbit reticulocyte lysate; wheat germ extract), which are commercially available.

We prepared mRNA from soybean seeds (Utsumi *et al.* 1987b) as described below. Midmaturation-stage soybean cotyledons were crushed in liquid N_2 using a coffee mill. Total RNA was extracted from the cotyledon powder at 0°C with an extraction buffer containing vanadyl–ribonucleoside complexes. The extract was treated with phenol. Polyadenylated RNA (mRNA) was prepared from the total RNA by oligo(dT)–cellulose column chromatography. The polyadenylated RNA was fractionated by centrifugation on a 10–35% (w/v) linear sucrose gradient. After centrifugation, the gradient was divided into fractions and analyzed by wheat germ cell-free translation.

2. *Synthesis and Cloning of cDNA*

Synthesis of cDNAs containing the whole coding sequences was very difficult because of the low purity of enzymes employed for cDNA synthesis, the low

transformation efficiency (using plasmids as a vector), and the method employed for the second-strand synthesis, based on self-priming and S1 nuclease digestion. However, a new method for the second-strand synthesis developed by Okayama and Berg (1982) and the availability of enzymes that are free from contaminating RNase made it possible to synthesize efficiently full-length cDNA encoding a large protein. Gubler and Hoffman (1983) modified and simplified the method of Okayama and Berg. The most efficient method to synthesize (nearly) full-size cDNA based on the method of Gubler and Hoffman is briefly described in Fig. 8. cDNA synthesis kits based on this method are available from many manufacturers. The cDNA first-strand synthesis is primed by oligo(dT) and catalyzed by reverse transcriptase. The second-strand synthesis is achieved by nicktranslation repair of the cDNA:mRNA hybrid, mediated by *E. coli* DNA polymerase I, RNase H, and *E. coli* DNA ligase (Okayama and Berg, 1982). This is the most valuable method to synthesize full-length cDNA. The T4 polynucleotide kinase phosphorylates the 5'-OH of the oligo(dT) primer (Sambrook *et al.*, 1989).

There are several methods for inserting cDNAs into vectors (Fig. 9). The homopolymer tailing method can be used only with plasmids, and in this case the length of homopolymer is important (~20 dC/dG residues on cDNAs and vectors) (Sambrook *et al.*, 1989). Before ligation of linkers to cDNAs, the cDNAs should be methylated to resist cleavage by the restriction enzyme specific for

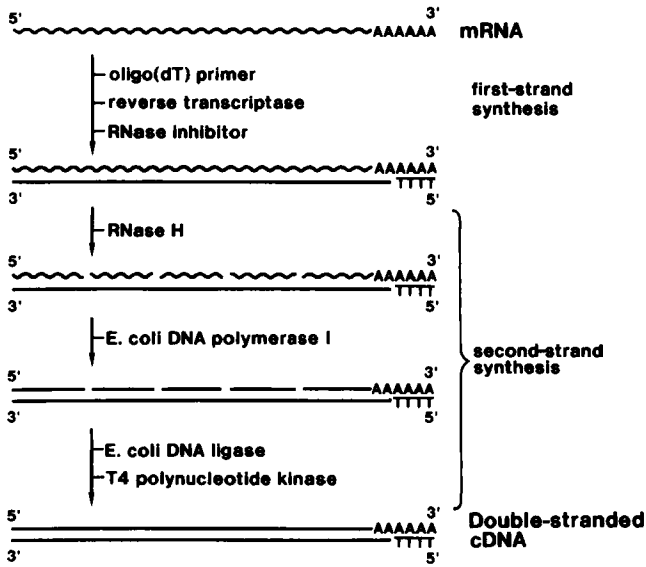


FIG. 8. Synthesis of double-stranded cDNA. See text for details.

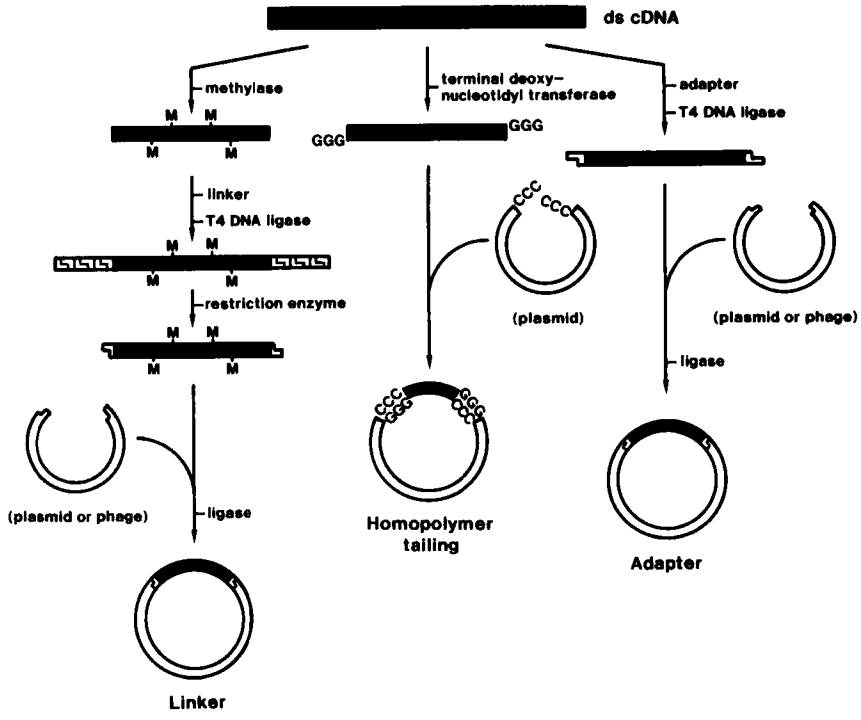


FIG. 9. Methods available for insertion of cDNA into vector. See text for details.

the recognition site involved in the linkers. Linkers and adapters can be used with both plasmids and λ vectors, and are available commercially.

The recombinant plasmids are transferred into *E. coli*-competent cells directly whereas the recombinant λ phages are transferred into *E. coli* by means of *in vitro* packaging (formation of phage particle). Competent cells and *in vitro* packaging systems with high efficiencies are commercially available.

We synthesized plasmid-primed double-stranded cDNAs from the glycinin mRNA-rich fraction (Utsumi *et al.*, 1987b) according to the procedure of Okayama and Berg (1982) and transformed them into *E. coli* by the method of Hanahan (1983).

D. GENOMIC DNA CLONING

Genomic DNAs are usually isolated from seedlings or young leaves by using proteinase K, urea–phenol–sarcosyl, or Triton X-100–sarcosyl methods (Malmberg *et al.*, 1985). By these methods, high-molecular-weight DNA suitable for the construction of a genomic DNA library can be prepared. Isolated genomic

DNAs are partially digested by a restriction enzyme, such as *Eco*RI, to produce 10- to 20-kb fragments. These fragments are then ligated with the right and left arms of phage vectors (Table XXIII) and the resultant bacteriophage is packaged *in vitro* into phage particles. Appropriate *E. coli* cells are transfected with the resultant phage particles.

E. SCREENING OF cDNA AND GENOMIC DNA LIBRARIES

The following three procedures are used to identify recombinant plasmids or phages carrying DNA encoding the target protein from libraries: (1) hybridization with probe DNA, (2) immunological screening using antibodies specific to the target protein, and (3) detection of function of the target proteins. The first two procedures can be employed, since seed storage proteins generally have no enzymatic function.

If cDNA encoding a part of the target protein or a related protein is available, it can be used as a hybridization probe to screen cDNA and genomic DNA libraries. Short oligonucleotides (17–20 mer) containing all possible sequences deduced from the amino acid sequences of the target proteins are powerful probes to screen the libraries. It is preferable that there are no more than 32 possible sequences. Amino acid sequences that are rich in amino acids coded by one (Met and Trp) or two (Phe, Tyr, Cys, Gln, Asn, Lys, Asp, and Glu) kinds of codons should be selected for screening. These probe DNAs are 5′-end labeled with ^{32}P and are used in colony (plasmids) or plaque (phage) hybridizations (Sambrook *et al.*, 1989).

If antibodies specific to the target proteins are available, they can be used as probes to screen cDNA expression libraries. Expression libraries can be constructed with pUC series vectors and λgt11. Antibodies should be purified by affinity chromatography on protein A–Sepharose, antigen–Sepharose, or *E. coli* proteins–Sepharose columns to extrude antibodies that interact with *E. coli* proteins. Detection of the target protein expressed in *E. coli* is performed according to the following procedures: (1) replication of plate to nitrocellulose filters, (2) incubation of the nitrocellulose filters with antibodies, (3) incubation of the nitrocellulose filters with second antibodies conjugated to horseradish peroxidase or alkaline phosphatase, and (4) color development with substrates of horseradish peroxidase or alkaline phosphatase. These detection systems are commercially available.

F. SEQUENCING OF DNA

Two sequencing techniques are currently employed, the enzymatic methods developed by Sanger *et al.* (1977) and the chemical method developed by Maxam and Gilbert (1977). The enzymatic method, the dideoxy-mediated chain-termination method, is much easier than the method of Maxam and Gilbert.

The procedures for the dideoxy-mediated chain-termination method are as follows: (1) preparation of a single-stranded DNA template, (2) annealling of primer with the DNA template, (3) dividing the annealled sample into four tubes for four kinds of dNTP, (4) synthesis of the DNA strand in the presence of [α-^{32}P]dCTP, four kinds of dNTP, and one of four ddNTPs in each tube by DNA polymerase (Klenow fragment of *E. coli* DNA polymerase I, T7DNA polymerase, or *Taq* DNA polymerase), and (5) electrophoresis of each sample under the conditions that can separate individual DNAs that differ in length by as little as one nucleotide (Sambrook *et al.*, 1989).

The target DNA is subcloned into bacteriophage M13mp18/M13mp19 or pUC118/pUC119 vectors. In each pair of vectors, the vector members have the same polycloning sites, but in the opposite direction. A single-stranded DNA template can be isolated from bacteriophage particles containing one strand of recombinant M13 or pUC. The 2′,3′-ddNTPs lack a hydroxyl residue at the 3′ position. These can be incorporated into a DNA strand synthesized by DNA polymerase, but the synthesis of DNA stops at the incorporation of ddNTP. DNA chains with different lengths are separated by urea–polyacrylamide gel and detected by autoradiography (Sambrook *et al.*, 1989).

To complete the sequencing of the target DNA, three strategies are possible: (1) Random digestion—the target DNA is digested with several restriction enzymes and the resultant DNA fragments are subcloned into M13 or pUC. (2) Deletion mutants—the target DNA is digested with Bal31 for different lengths of time. The resultant deleted fragments with different sizes are cloned into M13 or pUC. (3) Synthetic primer—the target DNA is cloned into M13 or pUC. The DNA sequence proximate to the universal primer site is determined. A new primer is synthesized from the determined sequence and is used for the next step of sequencing. This procedure is repeated until the whole sequence of the target DNA is determined.

Sequencing kits are commercially available.

VII. EXPRESSION OF CLONED GENES IN MICROORGANISMS

Production of engineered proteins is essential to investigate their capacity to form proper conformations and their functional properties. For this purpose, expression systems employing microrganisms such as *E. coli* and yeast are superior to those of cultured plant cells and transgenic plants because of the shorter time required for high-level production and ease of gene manipulation, although processing and modifiction cannot occur in *E. coli* and are restricted in yeast (Table XXV). *Escherichia coli* cannot splice mRNA precursors containing regions of introns. Yeast can splice mRNA precursors, but not precisely. Intron-free genes encoding proteins such as prolamin can be used for their

TABLE XXV

COMPARISON OF EXPRESSION SYSTEMS

Factor	*E. coli*	Yeast	Plant
Time required for production	Very short (1–2 days)	Short (2–3 days)	Very long (more than a few weeks)
Ease of gene manipulation	Very easy	Easy	Not easy
Processing			
Signal peptide	No[a]	Yes[a]	Yes
Proform to mature form	No[a]	No[a]	Yes
Modification	No[a]	Partly yes	Yes

[a]Note that exceptions exist.

expression in microorganisms. However, genes containing introns, such as globulin genes, cannot be used, and in this case cDNAs are essential for expression in microorganisms. Prior to protein engineering, establishment of a high-level expression system of the target proteins is required.

A. EXPRESSION IN *E. coli*

The system of protein synthesis in *E. coli* is well established, and this has led to the development of expression systems of foreign proteins in *E. coli*. The mechanisms and components required for protein synthesis are different in prokaryotes and eukaryotes. The 5′-untranslated sequences of the genes, including promoter regions and ribosome interaction sites, are quite different.

The essential units for gene expression in *E. coli* are illustrated in Fig. 10.

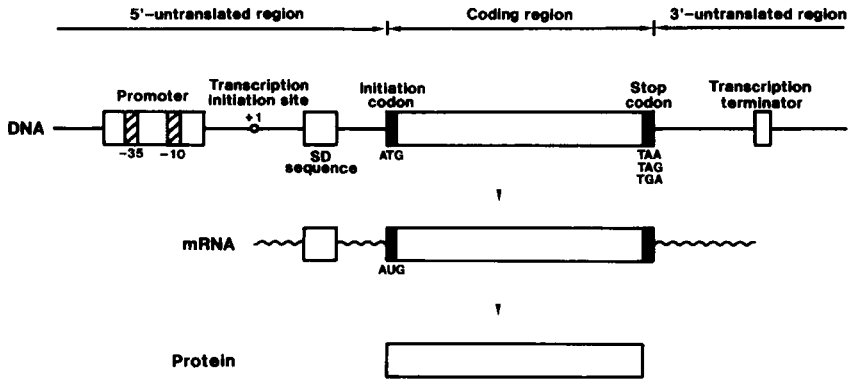


FIG. 10. The essential units for gene expression in *E. coli*. See text for details.

The promoter contains two consensus sequences, TATAAT (Pribnow box, −10 sequence) and TTGACA (−35 sequence). The former is located around 10 bp upstream and the latter around 35 bp upstream of the transcription initiation site. The Shine–Dalgarno (SD) sequence is a ribosome-binding site. The terminator functions to stop transcription. The translation initiation codon (ATG) and translation termination codon (TAA, TAG, and TGA) are necessary for initiating and terminating translation of mRNA to protein, respectively.

Many factors affect the levels of expression of foreign proteins in *E. coli* (Table XXVI). Strong promoters such as *lac*, *trp*, *tac*, P_L, and P_R are desirable for high-level expression. Suitable promoters for high-level expression vary, depending on the target proteins. An inducible promoter is sometimes important, because high-level expression of foreign proteins sometimes affects *E. coli* growth. Only one base difference in distance between the SD sequence and the initiation codon influences the expression level. Formation of a secondary structure around the promoter, the SD sequence, and the initiation codon inhibits transcription and/or translation. The 3′-noncoding region of the foreign gene should be short.

TABLE XXVI

FACTORS AFFECTING THE LEVEL OF EXPRESSION OF CLONED/MODIFIED GENES IN *E. COLI*

Factor	Reference
(1) Promoter	Nishimori *et al.* (1984)
(2) Distance and socondary structure between SD sequence and initiation codon	Matteucci and Heyneker (1983); Schoner *et al.* (1984); Kawaguchi *et al.* (1986); Sato *et al.* (1989)
(3) Sequence and secondary structure around initiation codon	Ganoza *et al.* (1978); Hui *et al.* (1984); Looman *et al.* (1987); Nobuhara *et al.* (1988); Utsumi *et al.* (1988a)
(4) Length of 3′-noncoding region	Utsumi *et al.* (1988a)
(5) Terminator	Gentz *et al.* (1981)
(6) Codon usage	Brinkmann *et al.* (1989)
(7) Host strain	Iwane *et al.* (1987); Sato *et al.* (1989); Utsumi *et al.* (1988a)
(8) Copy number of expression plasmid	Miki *et al.* (1987); Morino *et al.* (1988); Sato *et al.* (1989)
(9) Stability of expression plasmid	
(10) Stability of mRNA	Duvoisin *et al.* (1986)
(11) Stability of product	Stanley and Luzio (1984); Duvoisin *et al.* (1986); Utsumi *et al.* (1987a, 1988a)
(12) Culture condition (induction timing of expression, period of culture, temperature, speed of shaking, culture medium)	Kim *et al.* (1990a); Shirano and Shibata (1990)

Absence of the terminator causes instability of mRNA and plasmids. Codon usages in plant and *E. coli* genes are different. High contents of codons, which are rare in *E. coli*, cause low-level expression. The expression level of a certain protein is quite variable among *E. coli* strains. For example, tuna growth hormone was expressed at a level of 13% of total *E. coli* proteins in *E. coli* strain JM109, but was not expressed in C600, HB101, RB791, JM103, and JM105 (Sato *et al.*, 1989). The copy number of plasmids containing the replication origin of pBR322 is 15–20 per cell, but that of plasmids containing the replication origin of pUC vectors is 500–700 (Sambrook *et al.*, 1989). For a runaway vector, the copy number is >1000. These high-copy plasmids are usually useful for high-level expression. However, in the case of soybean glycinin, this was not observed (Kim *et al.*, 1990a). Unstable products are susceptible to proteinase digestion, resulting in very low-level accumulation in *E. coli*. Most products expressed at the high level form insoluble inclusion bodies in *E. coli* cells. Random association of expressed proteins caused by formation of unnatural disulfide bonds (Schoemaker *et al.*, 1985) and interaction with intracellular components (Darby and Creighton, 1990) are responsible for the formation of inclusion bodies, leading to high-level accumulation by escaping proteinase attack. Therefore, Stanley and Luzio (1984) attempted to make expressed proteins insoluble by producing them as fusion proteins. The signal peptide of soybean glycinin expressed in *E. coli*, because of its hydrophobicity, disturbs the folding of the expressed protein, rendering the expressed protein susceptible to proteinase digestion (Utsumi *et al.*, 1987a, 1988a). Culture conditions influence the expression level (Kim *et al.*, 1990a) and the solubility of the expressed proteins (Piatak *et al.*, 1988; Schein and Noteborn, 1988; Shirano and Shibata, 1990).

The factors indicated in Table XXVI are not uniform, but are variable among the target proteins. Therefore, the most suitable conditions for high-level expression should be assessed for each target protein.

Three types of expression vectors are commercially available (Table XXVII). Gene fusion vectors render high-level expression, accumulation, identification, and purification of the expressed proteins easy. However, it is essential to cleave the target protein from the fused protein by site-specific enzymes such as Factor Xa, which cleaves the C terminus of sequence Ile-Glu-Gly-Arg. This means that the recognition sequence of site-specific enzymes should be connected to the N-terminus of the target protein. Secretion vectors are useful to simplify the purification of the expressed proteins. However, seed storage proteins are transported into protein bodies, thus seed storage proteins expressed in *E. coli* are unlikely to be transported into the periplasmic space of *E. coli*. In fact, french bean phaseolin (Cramer *et al.*, 1985, 1987), soybean glycinin (Utsumi *et al.*, 1991), and pea vicilin (Watson *et al.*, 1988) expressed even in yeast were neither secreted into the periplasmic space or into the culture medium. Direct expression vectors are desirable for obtaining expressed proteins easily, if expressed proteins can accumulate in *E. coli* at high levels.

TABLE XXVII

CHARACTERISTICS OF *E. COLI* EXPRESSION VECTORS

Vector	Size (bp)	Promoter	Marker	Cloning site	Property	Manufacturer
Gene fusion vector						
pUC118/pUC119	2686	*lac*	Amp^r Blue/white colonies	10 recognition sites	Fusion with a part of β-galactosidase; inducible	Many manufacturers
pRIT2T	~4250	P_R	Amp^r	5 recognition sites	Fusion with protein A; easy purification; thermo-inducible	Pharmacia
pRIT5	~6900	*ProteinA*	Amp^r	5 recognition sites	Fusion with protein A; easy purification; translocation to periplasmic space	Pharmacia
pUEX1	6371	P_R	Amp^r	*Sma*I, *Bam*HI, *Sal*I, *Pst*I	Fusion with N-terminal 9 residues of *cro*-encoded protein and β-galactosidase; thermo-inducible	Amersham
pEX2	5775	P_r	Amp^r	5 recognition sites	Fusion with N-terminal 9 residues of *cro*-encoded protein and β-galactosidase; thermo-inducible	Boehringer, Clontech

pMAL-C	6145	*tac*	Amp^r	*Stu*I, *Eco*RI	Fusion with maltose-binding protein; easy purification; easy cleavage of the fusion protein with Factor Xa; inducible	New England Biolabs
pT3/T7-1	2700	T_3, T_7	Amp^r	10 recognition sites	Fusion with a part of β-galactosidase	Clontech
Secretion vector						
pKT279	~4300	*lac*	Amp^r	*Pst*I	Fusion with a signal peptide; translocation to periplasmic space; inducible	Clontech
Direct expression vector						
pKK223-3	4585	*tac*	Amp^r	6 recognition sites	Distance between SD sequence and ATG is changeable; inducible	Pharmacia
pKK233-2	4593	*tac*	Amp^r	*Nco*I, *Pst*I, *Hind*III	ATG initiation codon; inducible	Pharmacia, Clontech
pDR540	4063	*tac*	Amp^r	*Bam*HI	Inducible	Pharmacia
pDR720	~4100	*trp*	Amp^r	*Sal*I, *BAM*HI, *Sma*I	Inducible	Pharmacia
pPROK-1	~4600	*tac*	Amp^r	7 recognition sites	ATG initiation codon; inducible	Clontech

B. EXPRESSION IN YEAST

Yeast is a well-established eukaryote that is used for the expression of foreign proteins. Processing and modification of the expressed protein are possible in yeast but not in *E. coli*, although sometimes these events are not precise (Table XXV). Expression systems using *Saccharomyces cerevisiae* are widely used.

Yeast expression vectors are designed to be shuttle vectors that can replicate in both yeast and *E. coli*. Gene manipulation is carried out using *E. coli* and then the resultant expression plasmids are expressed in yeast. Yeast expression vectors (YEp plasmids, which replicate episomally in yeast) carrying the replication origin of 2-μm circular DNA are generally used, because such vectors are stable in yeast cells and their copy number is generally high (50–100 copies). Genetic markers such as *Trp1*, *Leu2*, *Ura3*, and *His4*, which complement auxotrophic mutants carrying *trp1*, *leu2*, *ura3*, and *his4*, are used for selection of yeast transformants. To achieve high-level expression, desirable expression vectors carry a strong promoter of the glycolysis enzyme genes, such as phosphoglycerate kinase (*PGK*) and glyceraldehyde 3-phosphate dehydrogenase (*GPD*). If the products are likely to be toxic to yeast, an inducible promoter, such as *PHO5* repressible acid phosphatase, is required. Other factors affecting the expression level are similar to those of *E. coli* (Table XXVI).

C. DETECTION OF EXPRESSED PROTEINS

Since seed storage proteins do not generally exhibit enzymatic activities, they are usually detected by immunological methods. There is a possibility that the expressed proteins will be secreted from the cells into the medium. Therefore, the cells and the medium should be analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), followed by immunoblotting. Briefly, in immunoblotting, the proteins separated on the gels are electrotransferred to nitrocellulose filters. The proteins immobilized on the filters are detected by their reaction with antibodies specific to the target proteins (Western blotting, immunoblotting), as described in Section VI,E. When the expression level is higher than 1% of the total bacterial proteins, the expressed protein is visible by Coomassie brilliant blue staining as compared with the electrophoretic pattern of the cells having vector only.

In the procedures described above, the detection of the products is carried out using two criteria, molecular size and immunoreactivity. To confirm this, determination of the N-terminal amino acid sequence of the probable target protein is required. Microsequencing methods have recently been developed in which very small amounts of protein separated by one-dimensional or two-dimensional PAGE are electroblotted onto membrane supports, such as 1,5-dimethyl-1,5-diazaundecamethylene polymethylbromide (Polybrene)-coated glass fiber filters (Vandekerckhove *et al.*, 1985), siliconized glass fiber filters (Eckerskorn *et al.*,

1988), and polyvinylidene difluoride (PVDF) membranes (Bauw *et al.*, 1987; Matsudaira, 1987), and are then sequenced directly with a gas-phase protein sequencer (Hirano and Watanabe, 1990). Using this method, the N-terminal amino acid sequence of the expressed protein can be easily determined without purification.

D. CHARACTERIZATION OF EXPRESSED PROTEINS

In order to employ the expression system for protein engineering, the expressed protein should fold in the correct conformation, including secondary, tertiary, and quaternary structures, and exhibit intrinsic properties such as solubility and functional properties similar to those of the native protein. These points should be investigated using standardized, conventional methods.

If the inherently salt-soluble protein forms inclusion bodies, the protein must be solubilized with a reducing agent and denaturant and then renatured. There are many reports that renaturation is very difficult, even if the native protein can renature from the reduced, denatured state. Darby and Creighton (1990) suggested that one reason for the insolubility of bovine pancreatic trypsin inhibitor (BPTI) expressed in *E. coli* was the formation of a complex with some other tightly bound substance. The BPTI purified by column chromatography under the extreme condition formed the same structure as the native BPTI (Darby and Creighton, 1990). Soybean glycinin expressed in *S. cerevisiae* exhibited similar behavior (Utsumi *et al.*, 1991). On the other hand, the renaturation of ovotransferrin from the reduced, denatured state having 15 intramolecular disulfide bonds was achieved by a two-step renaturation (Hirose *et al.*, 1989). These findings suggest that the renaturation of expressed proteins that aggregate to form inclusion bodies is possible using certain procedures, if they do not require special molecules, such as a molecular chaperon (Ellis and Hemmingsen, 1989), for their folding.

VIII. EXPRESSION OF SEED STORAGE PROTEINS IN MICROORGANISMS

Many eukaryotic proteins, including enzymes, have been successfully expressed at a high level in *E. coli* and *S. cerevisiae*, but the number of seed storage proteins expressed in these organisms is limited (Table XXVIII).

A. SOYBEAN GLYCININ

1. Escherichia Coli

Coding regions of the cDNAs encoding preproglycinin $A_{1a}B_{1b}$ and A_2B_{1a} subunits were placed under the control of the *trc* promoter in an expression vector

TABLE XXVIII

EXPRESSION OF SEED STORAGE PROTEINS IN MICROOGRANISMS

Gene	Origin	Host	Vector	Promoter	Expression level	Distribution	Solubility	Processing of signal peptide	Assembly	Reference
Glycinin										
Prepro$A_{1a}B_{1b}$	Soybean	*E. coli* JM105	pKK233-2	*trc*	Degrade	—[a]	—	—	—	Utsumi *et al.* (1987a)
PreproA_2B_{1a}	Soybean	*E. coli* JM105	pKK233-2	*trc*	Degrade	—	—	—	—	Utsumi *et al.* (1987a)
Prepro$A_{1a}B_{1b}$	Soybean	*E. coli* MV1190	pKK233-2	*trc*	1%	Cell	Insoluble	No	N.D.[b]	Fukazawa *et al.* (1987b)
PreproA_2B_{1a}	Soybean	*E. coli* MV1190	pKK233-2	*trc*	1%	Cell	Insoluble	No	N.D.	Fukazawa *et al.* (1987b)
Prepro$A_{1a}B_{1b}$	Soybean	Yeast AH22	pAM82	*PHO5*	0.3%[c]	Cell	Soluble	Yes	N.D.	Utsumi *et al.* (1988b)
Prepro$A_{1a}B_{1b}$	Soybean	Yeast AH22	pAM82	*PHO5*	3–5%	Cell	Insoluble	Yes	Yes	Utsumi *et al.* (1991)
Pro$A_{1a}B_{1b}$[d]	Soybean	*E. coli* JM105	pKK233-2	*trc*	0.1–2%	Cell	Soluble	—	Yes	Utsumi *et al.* (1988a)
Pro$A_{1a}B_{1b}$[d]	Soybean	*E. coli* JM105	pKK233-2	*trc*	20%	Cell	Soluble	—	Yes	Kim *et al.* (1990a)

Legumin[e]	Pea	Yeast MC16	pMA91	*PGK*	1–2%	Cell (golgi apparatus, ER)	Insoluble	N.D.	N.D.	Yarwood *et al.* (1987)
Legumin	Field bean	Yeast	?[f]	*ADH1*	?	Cell (vacuole)	?	?	?	Saalbach *et al.* (1990)
Vicilin[e]	Pea	Yeast MC16	pMA257	*PGK*	5%[c]	Cell	Soluble	—	Yes	Watson *et al.* (1988)
Phaseolin	French bean	Yeast JHC8-24C	YEp13	*Phaseolin*	0.01–0.03%	Cell	Soluble	Yes	N.D.	Cramer *et al.* (1985)
Phaseoline	French bean	Yeast W301-18A, 20B-12	pYE7	*PHO5*	3%[c]	Cell	Soluble	Yes	N.D.	Cramer *et al.* (1987)
HMW glutenin[d]	Wheat	*E. coli* BL21 (DE3)pLysS	pEt-3a	*lacUV5*	7%	Cell	Ethanol, 2-ME	—	Yes	Galili (1989)
α-Gliadin	Wheat	Yeast D1113-10B	pAY27	*CYC1*	0.1%	Cell	N.D.	Yes	—	Neill *et al.* (1987)
γ-gliadin	Wheat	Yeast XP660-19D	pYcDE-2	*ADH1*	40–80 μg/liter	Cell (partly secreted)	N.D.	N.D.	—	Scheets and Hedgcoth (1989)
Zein 19 kDa[e]	Maize	Yeast X4004-3A	pLGSD5	*CYC1*	5%	Cell (mitochondria)	Ethanol	—	—	Coraggio *et al.* (1986, 1988)
Zein 19 kDa	Maize	Yeast X4004-3A	Py46	*CYC1*	4%	Cell (ER)	Ethanol	50%	—	Coraggio *et al.* (1988)

[a]Experiment not possible.
[b]Not determined.
[c]As percentage of the soluble bacterial proteins. Other values are percentages of the total bacterial proteins.
[d]The DNA region corresponding to the signal peptide is deleted.
[e]See text.
[f]Not described.

pKK233-2 to construct expression plasmids pKG2($A_{1a}B_{1b}$) and pKG4(A_2B_{1a}) (Utsumi *et al.*, 1987a). The expression of these plasmids was examined in *E. coli* strains JM83, JM103, JM105, RB791, and MC1061 under different culture conditions (different media, temperatures, cultivation times, and induction timing). However, the expressed proteins were not detected by immunoblotting in the cells or the media. *In vitro* expression experiments using *E. coli* lysate (a commercial *in vitro* expression system) suggested that the expressed protein carrying the signal peptide could not fold to the correct conformation (Utsumi *et al.*, 1987a, 1988a).

Nucleotide sequences corresponding to the signal peptide and the mature N-terminal region were deleted stepwise from the cDNA encoding the preproglycinin $A_{1a}B_{1b}$ by Bal31 digestion, and the deleted cDNAs were placed under the control of the *trc* promoter in pKK233-2 (Fig. 11) (Utsumi *et al.*, 1988a). Since the translation initiation codon ATG was deleted from the cDNA by Bal31 digestion, the *Nco*I site (CCATGG) of the vector was filled in with the *E. coli* DNA polymerase I Klenow fragment after digestion by *Nco*I, repairing the ATG. The resultant plasmids were introduced into *E. coli* JM83. Sixteen plasmids having the deleted cDNA insert in the proper orientation were sequenced across the junctions between the initiation codon derived from the vector and the deleted cDNAs. The following seven in-frame constructs were obtained: mutants with 5′ end points at −15 (pKGA$_{1a}$B$_{1b}$5), −9 (pKGA$_{1a}$B$_{1b}$3), −3 (pKGA$_{1a}$B$_{1b}$1), 4 (pKGA$_{1a}$B$_{1b}$-1), 10 (pKGA$_{1a}$B$_{1b}$-3), 13 (pKGA$_{1a}$B$_{1b}$-4), and 34 (pKGA$_{1a}$B$_{1b}$-11) relative to the first letter of the mature N-terminal codon. The 3′ end points of the expression plasmids were tailored to that of pKGA$_{1a}$B$_{1b}$-3, because the most efficient expression was observed with pKGA$_{1a}$B$_{1b}$-3, which has 27 bp of 3′-noncoding region, among the seven expression plasmids in JM83.

pKGA$_{1a}$B$_{1b}$-3 was transferred into *lac*I^Q strains, JM105 and JM109, in which the *trc* promoter is inducible, and normal *lac*I strains AG1, MC1061, HB101, and JM83, in which the *trc* promoter is constitutive. Products obtained from each *E. coli* strain were obtained in quantities ranked as follows: AG1 > HB101 > JM105, JM109, JM83 > MC1061. The amount of product from AG1 was more than twice that from JM105. AG1, HB101, JM83, and MC1061, however, produced extra proteins, the sizes of which were about 3 kDa higher than the expected size of $A_{1a}B_{1b}$-3. This may have been caused by *supE*44, which is contained in those host cells, because the translation stop codon of the cDNA is an *amber* codon (TAG). Hence the following experiments were carried out using JM105. The type of stop codon should be considered for the expression of the target proteins.

Production of proglycinin homologue proteins in JM105 cells harboring individual expression plasmids was determined by densitometric analysis of the proteins following SDS–PAGE and immunoblotting (Table XXIX) (Utsumi *et al.*, 1988a). No expressed proteins from the cells harboring pKGA$_{1a}$B$_{1b}$Full and

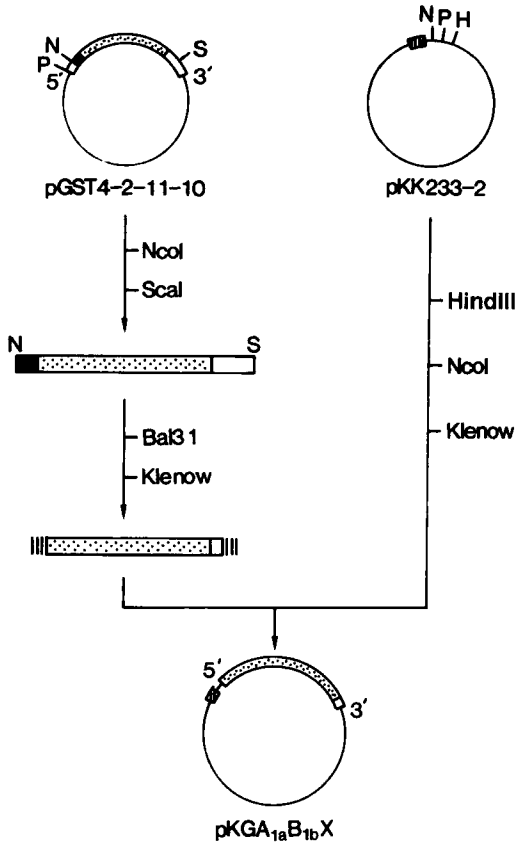


FIG. 11. Construction of expression plasmid $pKGA_{1a}B_{1b}X$. Open box, 3'- and 5'-noncoding regions of $A_{1a}B_{1b}$ cDNA; black box, signal sequence coding region of $A_{1a}B_{1b}$ cDNA; stippled box, proglycinin coding sequence; hatched box, *trc* promoter. H, N, P, and S denote the *Hin*dIII, *Nco*I, *Pst*I, and *Sca*I sites, respectively. (From Utsumi *et al.*, 1988a, with permission of the authors and publisher.)

$pKGA_{1a}B_{1b}5$ were detected in either the cells or the media. Proglycinin homologue proteins, however, accumulated as soluble proteins in the cells harboring the other expression plasmids. Dickinson *et al.* (1987), using an *in vitro* translation system, suggested that the folding and the self-assembly of preproglycinin were disturbed by the presence of a hydrophobic signal peptide. Together with the results obtained in an *in vitro* expression system, these results strongly suggest that the folding of the expressed products, with more than five amino acids of the signal sequence region, may be disturbed and this could make the proteins susceptible to proteinase digestion. Thus, the deletion of the signal peptide coding

TABLE XXIX

PRODUCTION OF $A_{1a}B_{1b}$ PROTEIN IN JM105[a]

Plasmid	Expressed protein[b]	
	mg/liter	% of total *E. coli* proteins
$pKGA_{1a}B_{1b}$Full[c]	N.D.[d]	—
$pKGA_{1a}B_{1b}5$	N.D.	—
$pKGA_{1a}B_{1b}3$	0.1–0.2	0.02–0.04
$pKGA_{1a}B_{1b}1$	0.5–1.0	0.1–0.2
$pKGA_{1a}B_{1b}$-1	0.5–1.0	0.1–0.2
$pKGA_{1a}B_{1b}$-3	5.8–9.3	1–2
$pKGA_{1a}B_{1b}$-4	0.5–1.0	0.1–0.2
$pKGA_{1a}B_{1b}$-11	2.1–3.2	0.4–0.6

[a]Data from Utsumi *et al.* (1988a), with permission of the authors and publisher.

[b]JM105 cells harboring individual expression plasmids were cultured in 600 ml for 15 hr at 30°C. The level of production was determined by densitometric analysis following immunoblotting after SDS–PAGE.

[c]The cDNA carried in $pKGA_{1a}B_{1b}$Full encodes the whole signal peptide region.

[d]Not detectable.

region from the glycinin cDNA is essential for cDNA expression in *E. coli*. This is, however, not in agreement with the results obtained by Fukazawa *et al.* (1987b), who observed that preproglycinin was not degraded and accumulated in MV1190. This may be due to the difference in *E. coli* strains, because Fukazawa *et al.* (1987b) used the same vector, pKK233-2.

The degree of accumulation of the products varied markedly depending on the expression plasmids used (Table XXIX) (Utsumi *et al.*, 1988a). Looman *et al.* (1987) investigated the influence of the codon following the ATG initiation codon on the expression of a modified *lacZ* gene in *E. coli* and demonstrated that the efficiency of gene expression varies by a factor of at least 15, depending on which codon follows the initiation codon. The results shown in Table XXIX basically support their proposal.

The expressed proteins from individual expression plasmids were blotted onto PVDF membranes and their N-terminal amino acid sequences were determined according to the procedure of Matsudaira (1987) (Fig. 12) (Utsumi *et al.*, 1988a). The N-terminal methionine was retained in $A_{1a}B_{1b}$-3 and -4, but was cleaved in $A_{1a}B_{1b}3$, 1, -1, and -11. Thus, the initiation methionine was processed from the synthesized proteins according to the proposal of Sherman *et al.* (1985) that the

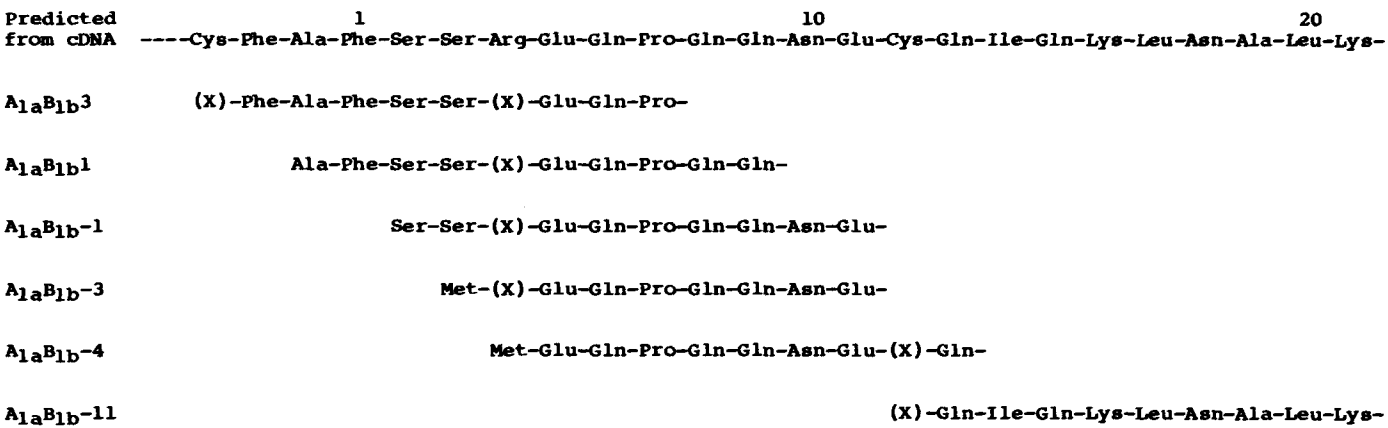


FIG. 12. The N-terminal amino acid sequences of the products from the expression plasmids shown in Table XXIX. The amino acids in parentheses (X) could not be determined. (From Utsumi *et al.*, 1988a, with permission of the authors and publisher.)

specificity of methionine amino peptidase is determined by the size of the side chain of the penultimate residue.

pKGA$_{1a}$B$_{1b}$-3 was the most suitable expression plasmid, the expressed protein being 1–2% of the total *E. coli* proteins (Table XXIX). This level of expression is not enough to carry out protein engineering on glycinin. An increase in the level of A$_{1a}$B$_{1b}$-3 expression was attempted by increasing the copy number of plasmids using a runaway vector (Uhlin *et al.*, 1979) or the replication origin of pUC9 (Miki *et al.*, 1987) and by changing the distance between the SD sequence and the initiation codon. However, these did not increase the level of A$_{1a}$B$_{1b}$-3 expression (Kim *et al.*, 1990a). Then the culture conditions for JM105, harboring pKGA$_{1a}$B$_{1b}$-3, were changed. The culture temperature and shaking speed were critical factors for high-level expression. Cultivation of the cells at 30°C with a shaking speed of 175 strokes/min gave an expression level of 1–2% of the total bacterial proteins. However, the optimum temperature and shaking speed for high-level expression were, respectively, 37°C and 85–90 strokes/min, resulting in an expression level of 20% of the total bacterial proteins (Kim *et al.*, 1990a). Even at such a high level, the expressed protein was soluble and did not form inclusion bodies.

In order to investigate the structure and properties of the expressed protein (A$_{1a}$B$_{1b}$-3), it was purified by ammonium sulfate fractionation, Q-Sepharose column chromatography, and cryoprecipitation (an intrinsic property of glycinin) (Kim *et al.*, 1990a). The purified A$_{1a}$B$_{1b}$-3 predominantly occurred as trimers, as judged from sucrose density gradient centrifugation (Kim *et al.*, 1990a). The secondary structure of the trimers of the A$_{1b}$B$_{1b}$-3 was similar to that of the glycinin half-molecule (Utsumi *et al.*, 1987c; Kim *et al.*, 1990a). These facts suggest that the A$_{1a}$B$_{1b}$-3 expressed in *E. coli* self-assemble to a trimer with a structure similar to that of the glycinin half-molecule, but not the hexamer, since processing of the junction between the acidic and basic polypeptide regions is necessary for the assembly to a hexamer (Dickinson *et al.*, 1989). Cryoprecipitation (Wolf and Sly, 1967) and calcium-induced precipitation (Wolf and Briggs, 1959; Saio *et al.*, 1973) are fundamental intrinsic properties of glycinin. A$_{1a}$B$_{1b}$-3 exhibited these properties and in fact it can be purified by cryoprecipitation (Kim *et al.*, 1990a). Moreover, A$_{1a}$B$_{1b}$-3 had the functional properties of heat-induced gelation (Kim *et al.*, 1990a) and emulsification (Kim *et al.*, 1990b). These observations indicate that the *E. coli* expression system of glycinin cDNA may be used for the protein engineering of glycinin.

2. *Saccharomyces Cerevisiae*

The cDNA sequence encoding the preproglycinin A$_{1a}$B$_{1b}$ subunit containing 3′- and 5′-noncoding regions was placed under control of the repressible acid phosphatase promoter *PHO5* of the yeast *S. cerevisiae* in an expression vector

pAM82 (Utsumi *et al.*, 1991). $pAMA_{1a}B_{1b}SO$, $pAMA_{1a}B_{1b}S1$, and $pAMA_{1a}B_{1b}S2$ expression plasmids were constructed, each of which had a different length of the 3′-noncoding region of $A_{1a}B_{1b}$ cDNA. $pAMA_{1a}B_{1b}SO$ and S1 contain approximately one-fifth of the 3′-noncoding and two-thirds of the 3′-noncoding regions, respectively, while $pAMA_{1a}B_{1b}S2$ contains the whole 3′-noncoding region and poly(A). The resultant plasmids were introduced into a yeast recipient strain, AH22, selecting for Leu^+ colonies. The amount of product obtained from each expression plasmid was ranked as follows: $pAMA_{1a}B_{1b}S1$ (100%) > $pAMA_{1a}B_{1b}S2$ (~30%) > $pAMA_{1a}B_{1b}SO$ (~5%). Together with the fact that the expression vector pAM82 has no transcription terminator downstream of *PHO5* promoter, this suggests that the 3′-noncoding region of the cDNA carried in $pAMA_{1a}B_{1b}S1$ but not in $pAMA_{1a}B_{1b}SO$ functions as a transcription terminator. The function of this region could be inhibited by the sequence that is present in $pAMA_{1a}B_{1b}S2$ but not in $pAMA_{1a}B_{1b}S1$. The protein expressed from $pAMA_{1a}B_{1b}S1$ is composed of ~5% of the total yeast proteins, or 30–40 mg per liter of culture. The $A_{1a}B_{1b}$ protein was not detected in the media, indicating that this protein, expressed in yeast, was not secreted into the media.

Of the expressed proteins, 10–15% and 85–90% were observed in the soluble and the insoluble fractions, respectively (Utsumi *et al.*, 1991). The N-terminal sequences of the expressed proteins in the soluble (Utsumi *et al.*, 1988b) and insoluble (Utsumi *et al.*, 1991) fractions were determined to be Phe-Ser-Ser-(X)-Glu-Gln-Pro-Gln-Gln-Asn-Glu-. This is consistent with the N-terminal sequence of the mature A_{1a} polypeptide predicted from the nucleotide sequence of $A_{1a}B_{1b}$ cDNA (Utsumi *et al.*, 1987b). Thus, the signal sequences of both the soluble and insoluble expressed proteins were cleaved at the same processing site as in soybean. The size of the expression protein was found to be ~57 kDa, indicating that the expressed protein is in the proform.

The signal sequence of the expressed preproglycinin is correctly recognized and processed at the same site as in soybean by the yeast processing system, as described above. This indicates that the expressed protein is transferred into the endoplasmic reticulum, and is either retained in the endoplasmic reticulum or is transferred to the golgi apparatus, vacuole, and/or periplasmic space. Cellular distribution of the expressed proteins from $pAMA_{1a}B_{1b}S1$ was demonstrated by immunocytochemical localization in sections cut by cryoultramicrotomy (Fig. 13) (Utsumi *et al.*, 1991). Most of the amorphous materials were associated with a golgi-like structure that was similar to that observed by Semenza *et al.* (1990), and with a certain organelle that seems to be derived from the golgi-like structure or a vacuole that has properties different from a normal vacuole. Some additional staining was also associated with an endoplasmic reticulum-like structure, which resembled that observed by Yarwood *et al.* (1987).

The expressed protein in the insoluble fraction was solubilized in a 35 m*M* K–P_i buffer (pH 7.6) containing 6 *M* urea and 0.2 *M* 2-mercaptoethanol. When

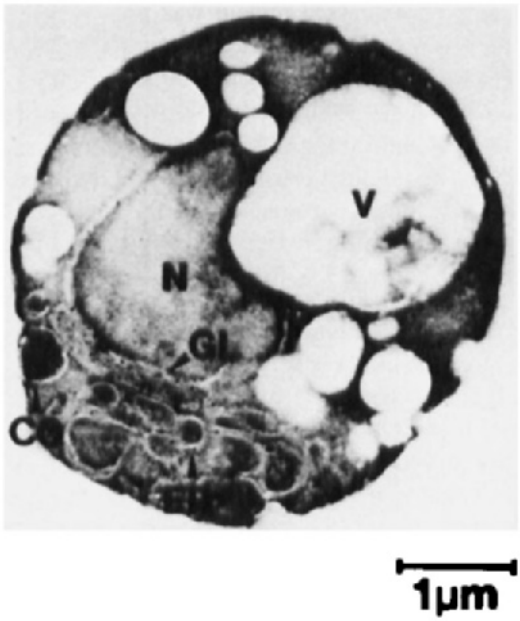


FIG. 13. Immunogold labeling of yeast harboring pAMA$_{1a}$B$_{1b}$S1. N, Nucleus; V, vacuole; GL, golgi-like structure; ER, endoplasmic reticulum-like structure; CO, a certain organelle. (From Utsumi *et al.*, 1991, with permission of the authors and publisher.)

the solubilized protein was dialyzed against a 35 m*M* K–P_i buffer (pH 7.6) containing 0.4 *M* NaCl, the expressed protein again precipitated (Utsumi *et al.*, 1991). However, the expressed proteins purified by Q-Sepharose column chromatography in the presence of 6 *M* urea and 0.2 *M* 2-mercaptoethanol were soluble after dialysis against the buffer described above, and formed trimers. The acidic polypeptide region expressed in yeast from the cDNA encoding the acidic polypeptide region, which was constructed by deletion of the cDNA region corresponding to the basic polypeptide region from the A$_{1a}$B$_{1b}$ cDNA, was also insoluble. These results suggest that the interaction of the acidic polypeptide region with some intracellular component caused the insolubilization of the A$_1$B$_{1b}$ protein expressed in yeast.

B. PEA LEGUMIN

A hybrid cDNA encoding legumin A was constructed from several shorter cDNAs and the *LegA* gene, and was placed under the control of the *PGK* promoter in expression vector pMA91 to construct expression plasmid pJY21 (Yarwood *et al.*, 1987). The resultant plasmid was introduced into *S. cerevisiae* strain MC16, selecting for Leu$^+$ colonies. The expression level was 1–2% of the total

yeast proteins, and the expressed protein was predominantly observed in the insoluble fraction and was almost undetectable in the soluble fraction. The size of the expressed protein was approximately 60 kDa, indicating that processing of the junction between the acidic and basic polypeptides did not occur. Immunogold labeling demonstrated that the expressed protein was predominantly accumulated within golgi vesicles, and some was accumulated in the endoplasmic reticulum.

C. PEA VICILIN

A cDNA encoding the vicilin 50-kDa subunit was placed under control of the *PGK* promoter in expression vector pMA257 to construct expression plasmid pDUB2018 for the expression of a fusion protein composed of the N-terminal 16 amino acid residues from phosphoglycerate kinase, three residues from the *Bam*HI linker, and three residues from the putative vicilin signal sequence and then the mature vicilin sequence (Watson *et al.*, 1988). This plasmid was transformed into *S. cerevisiae* strain MC16, selecting for Leu^+ colonies. The expressed protein was soluble and present in the cell. The expression level was approximately 5% of the yeast total proteins. The expressed protein was purified by ammonium sulfate fractionation and HA-Ultrogel column chromatography. Assembly of the purified protein was analyzed by gel filtration, and it was observed that a part of the protein assembled into trimers but majority were monomers, dimers, and hexamers. This minimal ability of the expressed proteins to assemble into trimers may have been due to the presence of an additional 22 N-terminal amino acids.

D. FRENCH BEAN PHASEOLIN

A 3.8-kb genomic DNA fragment containing the phaseolin coding sequence and 5′- and 3′-flanking sequences was introduced into *S. cerevisiae*, but the size of transcript from the gene was much smaller than expected and no phaseolin polypeptide was detected (Cramer *et al.*, 1985).

A Chimeric DNA composed of the phaseolin coding sequence (α or β) of cDNAs and 5′-flanking (800 bp) and 3′-flanking (120 bp) sequences of a genomic DNA was constructed and was placed in yeast vector YEp13 (Cramer *et al.*, 1985). The resultant plasmid carrying the phaseolin promoter was introduced into *S. cerevisiae* strain JHC8-24C, selecting for Leu^+ colonies. The expressed protein present in the cells was soluble, and the expression level was 0.01–0.03% of the total cellular proteins. The expressed protein was N-glycosylated similarly to phaseolin in french bean, although the nature and location of carbohydrate moiety were not determined. The size of the unglycosylated protein was consistent with the size expected after removal of the signal peptide, suggesting that signal peptide processing occurs in yeast.

A phaseolin cDNA was placed under control of *PHO5* promoter in pYE7 to construct expression plasmid pYE7ph (Cramer *et al.*, 1987). The resultant plasmid was introduced into *S. cerevisiae* strains W301-18A and 20B-12. The expressed protein was present in the soluble fraction of yeast extracts, and the expression level was approximately 3% of the total soluble cellular proteins. This indicates that using the yeast promoter is superior to using the plant promoter for high-level expression. The expressed proteins were N-glycocylated and their sizes suggested that the signal peptide was processed. Phaseolin polypeptides from the cDNA-deleted region corresponding to the signal sequence were not posttranslationally modified in yeast.

E. WHEAT HMW GLUTENIN

A HMW glutenin subunit 1Dx2 coding region without the signal peptide coding region of genomic DNA was placed under the control of the *lacUV5* promoter in *E. coli* expression vector pEt-3a to construct expression plasmid pEt-3a-Glu-1Dx2 (Galili, 1989). The resultant plasmid was introduced into *E. coli* Bl21(DE3)pLysS. The expressed protein was in the cells and was soluble in 70% alcohol containing 1% 2-mercaptoethanol. The expression level was approximately 7% of the total *E. coli* proteins. The expressed protein solubilized in alcohol solution containing 2-mercaptoethanol and was nearly homogeneous and exhibited charge heterogeneity (two or three charged isomeric forms). Oligomer formation of the expressed protein through intermolecular disulfide bonds was revealed by SDS–PAGE.

F. WHEAT α-GLIADIN

A portion of a genomic wheat α-gliadin gene consisting of the coding sequence and 18 bp of 5′- and 54 bp of 3′-noncoding sequences was placed under the control of the promoter of the iso-1-cytochrome *c* (*CYC1*) gene in expression vector pAY27 to construct expression plasmid pAY31 (Neill *et al.*, 1987). The resultant plasmid was transferred into *S. cerevisiae* strain D1113-10B. The expressed protein was present in the cells, and its size was consistent with that of the mature α-gliadin, suggesting that the signal peptide of the expressed protein was cleaved. The expression level was approximately 0.01% of the total yeast proteins.

G. WHEAT γ-GLIADIN

A complete γ-gliadin coding region, including the signal peptide coding region, was inserted into a yeast expression vector containing the *ADH1* promoter and *CYC1* transcription terminator to construct expression plasmid pY2Gc (Scheets and Hedgcoth, 1989). The resultant plasmid was introduced into *S. cerevisiae*

strain XP660-19D, selecting for Trp^+ colonies. Most of the expressed protein was in the cells and the expression level was 40–80 μg/liter of culture.

H. MAIZE ZEIN

The 19- and 21-kDa zein genes containing coding sequences and promoters were inserted into yeast vector YIp35C (Langridge *et al.*, 1984). The resultant plasmids were introduced into *S. cerevisiae* strain VB2-20A, selecting for Ura^+ colonies. The transcripts of the plasmids were detected and their synthesis started at the same nucleotide positions used in maize.

A 19-kDa zein genomic DNA carrying a coding sequence and 5′- and 3′-flanking sequences was inserted into yeast vector pLGSD5 with a yeast upstream activator site (UAS) to construct expression plasmid pL15 (Coraggio *et al.*, 1986). The resultant plasmid was introduced into *S. cerevisiae* strain X4004-3A, selecting for Ura^+ colonies. The transcription from this plasmid occurred correctly.

A 19-kDa zein genomic DNA fragment was placed under the control of the *CYC1* promoter in vector pLGSD5 to construct expression plasmid pL27, which expresses a fusion protein composed of the N-terminal four amino acid residues derived from the construction and the 19-kDa zein deleted mature N-terminal 36 residues (Coraggio *et al.*, 1986). The expressed protein from this plasmid was in the yeast cells (primarily in mitochondria) and was soluble in alcohol solution (Coraggio *et al.*, 1986, 1988). The expression level reached approximately 5% of the total yeast proteins under the best cultivation conditions. When the 19-kDa zein whole coding sequence, including the signal peptide coding region, was placed under control of the *CYC1* promoter in yeast vector Py46 to construct expression plasmid Py19K, the plasmid expressed the zein protein at a level of about 4% of the total yeast proteins. The signal peptide of the expressed protein was partly processed and the expressed protein accumulated in the endoplasmic reticulum (Coraggio *et al.*, 1988).

IX. EXPRESSION OF SEED STORAGE PROTEINS IN TRANSGENIC PLANTS

Development of transgenic plants that produce modified proteins is required for evaluating whether the modified proteins can undergo posttranslational modifications and accumulate in the protein bodies of the seeds in a manner similar to that of the native proteins, while exhibiting the desired functional properties. Such achievements in transgenic crops are necessary to enhance their production as food resources. To get transgenic plants, transformation of modified genes into plant cells or explants, regeneration of whole plants, and specific and high-level expression of modified genes in seeds are required.

A. TRANSFORMATION AND REGENERATION OF PLANTS

Progress in transformation techniques and the totipotency of plant cells and explants make possible the regeneration of transgenic plants. Two general methods of DNA transfer into plant cells or explants have been developed. One method is the *Agrobacterium tumefaciens*-mediated transformation method using a tumor-inducing (Ti) plasmid; the other approach involves direct transformation methods, including DNA-coated microprojectiles, microinjection of DNA, and uptake of DNA into protoplasts stimulated either electrically (electroporation) or chemically (Cocking, 1990).

1. Agrobacterium tumefaciens-Mediated Transformation System

The gram-negative bacterium *A. tumefaciens* carries the Ti plasmid. When *A. tumefaciens* infects plants (mainly dicots) by entry through wound sites, transferred DNA (T-DNA) from the Ti plasmid integrates into plant nuclear DNA. T-DNA encodes enzymes involved in the biosynthesis of auxin and cytokinin. Integration of T-DNA leads to the constitutive synthesis of the phytohormones, resulting in tumor inducement. Two regions of the Ti plasmid are essential for the mobilization and integration of the T-DNA into plant cells: (1) the T-DNA border sequences (left and right borders), which consist of direct repeats of 25 bp, and (2) the virulence (*vir*) region, which is required for the excision, transfer, and integration of the T-DNA from the Ti plasmid into the plant genome (Wu, 1989). The virulence gene acts in trans. Substitution of the DNA region encoding phytohormones with the target gene makes possible the introduction and integration of the target gene into the plant nuclear DNA without inducing tumors. As a marker for selection of transformant, a selectable marker gene, such as a bacterial antibiotic resistance gene, should be introduced with the target gene.

Two types of shuttle vectors (recombinant DNA can be constructed using *E. coli* and can be transferred into plants through *A. tumefaciens*) are developed from the Ti plasmid: an integrating vector and a binary vector (Horsch *et al.*, 1988).

The leaf disk techniques (Horsch *et al.*, 1985) is widely used for the introduction of other *A. tumefaciens* into explants. In this method, gene transfer, selection, and regeneration are coupled together, and any responsive explant can be used (Horsch *et al.*, 1988).

2. Direct Transformation System

The *Agrobacterium tumefaciens*-mediated transformation system is useful for most dicots but is unsuitable for monocots. A direct transformation system is employed for transforming monocots.

Electroporation (Fromm *et al.*, 1986) and polyethylene glycol (PEG) (Paszkowski *et al.*, 1984) methods are used for transforming protoplasts. PEG and electrical impulses stimulate the uptake of DNA molecules into protoplasts. However, regeneration of whole plants from protoplasts is usually difficult and requires special techniques (Wu, 1989).

In the microinjection method (Crossway *et al.*, 1986), the target gene is directly introduced into intact cells or organs under the microscope using a microcapillary tube. However, it is possible to execute exact introduction of DNA into only a few cells, and this requires great skill.

A microprojectile bombardment method was recently developed. In this method, the DNA-coated microprojectiles (tungsten, gold) are injected using a particle gun (Klein *et al.*, 1987; McCabe *et al.*, 1988). This method requires skillful techniques and special equipment.

3. *Regeneration of Plants*

The cells or explants transformed with the target genes are cultured in liquid media or on agar media containing auxin and cytokinin. After shoot generation, they are transferred to agar media without auxin and cytokinin. The rooted plantlets are then transferred to soil.

Regeneration of the fertile transgenic plants of the family Solanaceae, including tobacco, *Petunia,* tomato, and egg plant, is easy, but regeneration of members of the families Leguminosae and Gramineae is usually difficult. By using the microprojectile bombardment method, fertile transgenic plants of soybean (McCabe *et al.*, 1988; Christou *et al.*, 1989) and maize (Gordon-Kamm *et al.*, 1990) have been achieved, but not to a level sufficient for breeding transgenic crops.

Exogenous DNA introduced into cells using any of the above-mentioned methods may be integrated into the genome via illegitimate recombination at random locations. Position effects result in differences in the expression level of the integrated DNA in different transformants. To avoid position effects, gene targeting in plant cells has been attempted (Paszkowski *et al.*, 1988; Offringa *et al.*, 1990).

B. DNA ELEMENT REQUIRED FOR REGULATION OF GENE EXPRESSION

To generate transgenic crops, the modified gene integrated into the nuclear DNA should be expressed in a manner similar to the native gene, having high-level, seed-specific, and developmental-specific expression. This demands elucidation of the regulation mechanism of the seed storage protein gene expression. The development of techniques required for the transformation of exogenous

genes in other plant species and regeneration of transgenic plants will help elucidate the mechanisms.

The cis-control elements required for the regulation of expression of various seed storage protein genes have been studied (Table XXX). The chloramphenicol acetyltransferase (CAT) gene and the β-glucuronidase (GUS) gene are frequently used as reporter genes to investigate cis-control elements. The gene encoding GUS has a higher sensitivity of detection than that CAT gene. All the exogenous genes that have been determined so far exhibited temporal and tissue-specific expression in transgenic tobacco and *Petunia*, similar to that in the original plants, indicating that trans-acting factors in tobacco and *Petunia* are common to or resemble those of the original plants. However, the positions and the sequences of cis-control elements in 5′-flanking sequences are not uniform among seed storage protein genes.

1. 7S Globulins

Deletion mutants containing the β-conglycinin α′-subunit gene, flanked in the 5′ direction from +14 nucleotides to −8.5 kb relative to the site of transcription initiation, were constructed and then introduced into the genome of *Petunia* cells using Ti plasmid-derived vectors (Chen *et al.*, 1986). The α′-subunit gene, flanked by 159 nucleotides upstream (Δ-159), was expressed at a low level but specifically in the seed. This indicates that the cis-control element required for tissue-specific expression is present in Δ-159, which contains the vicilin box (Table IX). The Δ-257 exhibited high-level expression, suggesting that the nucleotide sequence between Δ-257 and Δ-159 plays an important role in determining the expression level of the α′-subunit gene. In this sequence, four repeats of a 6-bp sequence

$$\begin{matrix} & \text{A} & \\ \text{AGCCCA} & & \\ & \text{C} & \end{matrix}$$

are present and Δ-159 contains one of the sequences. By using a chimeric reporter gene composed of the cauliflower mosic virus 35S promoter, the CAT gene, and the polyadenylation signal from the α′-subunit gene, Chen *et al.* (1988) confirmed that the nucleotide sequence corresponding to the region of −257 to −78 bp relative to the site of transcription initiation confers seed-specific enhancement. Nuclear proteins interacted with a region composed of two DNA elements located at −183 to −169 bp and −153 to −134 bp relative to the start of transcription (Allen *et al.*, 1989). Both elements include AACCCA. The nuclear proteins interacted with synthetic DNA containing the elements, but not with synthetic DNA containing AAGGCA. The binding activity was observed specifically in the seeds and was found to increase and decrease during seed

TABLE XXX
SPECIFIC EXPRESSION OF SEED STORAGE PROTEIN GENES IN TRANSGENIC PLANTS

			Temporal specificity		Tissue specificity					Cis-regulatory region				
Gene	Origin	Host	Specificity	Detection[a]	Seed	Leaf	Root	Stem	Detection[a]	Specificity	Detection	Enhancer	Detection	Reference
7S globulins														
β-Conglycinin α′	Soybean	*Petunia*	Y[b]	Protein	Seed	X[c]	N.D.[d]	N.D.	Protein	−159 ~ +1	Protein	−257 ~ −159	Protein	Beachy *et al.* (1985); Chen *et al.* (1986)
β-Conglycinin α	Soybean	Tobacco	Y	CAT	Seed	X	X	X	CAT	—	—	−257 ~ −78	CAT	Chen *et al.* (1988)
β-Conglycinin β	Soybean	Tobacco	Y	mRNA	Embryo	X	N.D.	N.D.	mRNA	N.D.	—	N.D.	—	Barker *et al.* (1988)
Phaseolin β	French bean	Tobacco	Y	Protein	Embryo	X	N.D.	N.D.	Protein	N.D.	—	N.D.	—	Sengupta-Gopalan *et al.* (1985)
Phaseolin β	French bean	Tobacco	Y	GUS	Embryo	X	X	N.D.	GUS	−782 ~ +20	GUS	−678 ~ −619	GUS	Bustos *et al.* (1989)
Vicilin	Pea	Tobacco	Y	Protein	Embryo	X	X	X	Protein	N.D.	—	N.D.	—	Higgins *et al.* (1988)
11S globulins														
Legumin A	Pea	Tobacco	Y	Protein	Seed	X	N.D.	N.D.	Protein	−549 ~ −97	Protein	−1203 ~ −549	Protein	Ellis *et al.* (1988); Shirsat *et al.* (1989)
Legumin A1	Pea	Tobacco	Y	Protein	Seed	X	X	X	Protein	−668 ~ +1	Protein	−668 ~ −237	Protein	Rerie *et al.* (1991)
Legumin B4	Field bean	Tobacco	N.D.	—	Seed	X	N.D.	X	Protein	−2400 ~ +1	NPT II[e], GUS	−1200 ~ −193	NPT II	Bäumlein (1988, 1991)
Glycinin $A_{1a}B_{1b}$	Soybean	Tobacco	Y	?[f]	Seed	X	N.D.	N.D.	?	−66 ~ +1	?	−464 ~ −66	?	Goldberg *et al.* (1989)
Helianthinin	Sunflower	Tobacco	Y	Protein	Seed	X	N.D.	N.D.	Protein	−2376 ~ +24	GUS	−725 ~ −322	GUS	Bogue *et al.* (1990) Jordano *et al.* (1989)
Glutelin Gt3	Rice	Tobacco	Y	CAT	Seed	Ldw	X	Low	CAT	−980 ~ +96	CAT	N.D.	—	Leisy *et al.* (1990)
Glutelin type II	Rice	Tobacco	Y	GUS	Endosperm	X	N.D.	X	GUS	−441 ~ −237	GUS	N.D.	—	Takaiwa *et al.* (1991)
Prolamins														
19-kDa zein	Maize	Sunflower, carrot	N.D.	—	N.D.	N.D.	N.D.	N.D.	—	N.D.	—	−337 ~ −125	mRNA, CAT	Roussell *et al.* (1988)
19-kDa zein	Maize	*Petunia*	Y	mRNA	Seed	Low	N.D.	Low	mRNA	−881 ~ +1	mRNA	ND.	—	Ueng *et al.* (1988)
19-kDa zein	Maize	Carrot	N.D.	—	N.D.	N.D.	N.D.	N.D.	—	N.D.	—	−309 ~ −226	GUS	Thompson *et al.* (1990)
19-kDa zein	Maize	*Petunia*	Y	GUS	Endosperm	X	Low	X	GUS	−430 ~ −7 −1415 ~ −430	GUS GUS	N.D.	—	Quattrocchio *et al.* (1990)
22-kDa zein	Maize	Tobacco	Y	GUS	Endosperm	X	X	X	GUS	−174 ~ +1	GUS	−886 ~ −174	GUS	Schernthaner *et al.* (1988); Matzke *et al.* (1990)
HMW glutenin	Wheat	Tobacco	Y	CAT	Endosperm	X	X	X	CAT	−433 ~ −5	CAT	N.D.	—	Robert *et al.* (1989)
HMW glutenin	Wheat	Tobacco	Y	GUS	Endosperm	X	N.D.	N.D.	GUS	−375 ~ −45	GUS	−186 ~ −148	GUS	Thomas and Flavell (1990)
HMW glutenin	Wheat	Tobacco	Y	GUS	Endosperm	X	X	X	GUS	−277 ~ +39	GUS	N.D.	—	Halford *et al.* (1989)
LMW glutenin	Wheat	Tobacco	Y	CAT	Endosperm	X	X	X	CAT	−326 ~ −160	CAT	N.D.	—	Colot *et al.* (1987)
α/β-Gliadin	Wheat	Tobacco	N.D.	—	N.D.	N.D.	N.D.	N.D.	—	N.D.	—	−592 ~ −448, −218 ~ −141	CAT	Aryan *et al.* (1991)
Hordein B1	Barley	Tobacco	Y	CAT	Endosperm	X	N.D.	N.D.	CAT	−512 ~ +37	CAT	N.D.	—	Marris *et al.* (1988)

[a] When the translation products were detected, in most of the cases mRNA was also detected.
[b] Yes.
[c] Not detectable or background level.
[d] Not determined.
[e] Neomycin phosphotransferase II.
[f] Not described.

development in a manner similar to that of β-conglycinin mRNA synthesis. This indicates that the AACCCA sequence functions as a seed-specific enhancer (Allen *et al.*, 1989).

The β-conglycinin β-subunit gene contains neither the 6-bp sequence element shown above nor the vicilin box (Harada *et al.*, 1989). This could be related to differences between the α′- and β-subunit genes in expression timing (Meinke *et al.*, 1981; Naito *et al.*, 1988) and strength of the promoter (Naito *et al.*, 1988). Lessard *et al.* (1991) observed that the binding sites for nuclear proteins in seed nuclear extracts are distributed unequally among the α′- and β-subunit genes.

The DNA region of −678 to −619 bp relative to the site of transcription initiation of the phaseolin β-subunit gene has a function of enhancer and is A/T rich (Bustos *et al.*, 1989). This DNA element also interacted with nuclear proteins. Transcription activation by this element was not restricted to the seeds, indicating that additional regulatory elements are required for embryo-specific gene expression (Bustos *et al.*, 1989).

2. *11S Globulins*

Deletion mutants of the pea legumin A subunit gene were constructed and transferred into tobacco using the Ti plasmid-derived vector (Shirsat *et al.*, 1989). A construct with 549 bp of upstream flanking sequence (Δ-549) was expressed at a low level, but preserved seed specificity and temporal regulation. The Δ-1203 was expressed at a significantly higher level than Δ-549, and the Δ-124, including the legumin box, failed to activate the gene and did not bind nuclear proteins (Shirsat *et al.*, 1989, 1990; Meakin and Gatehouse, 1991). Nuclear proteins bound to Δ-547 (Shirsat *et al.*, 1990) and to the −833 to −582 and −549 to −316 fragments (Meakin and Gatehouse, 1991). Moreover, Bäumlein *et al.* (1991) observed that the legumin box of the field bean legumin B4 gene had no obvious effects on expression level. Rerie *et al.* (1991) demonstrated that the nucleotide sequence located between positions −668 and −237 relative to the site of transcription initiation of the pea legumin A gene was essential for the high level of regulated gene expression. These results indicate that the legumin box is not the sole promoter determining the legumin gene expression (Shirsat *et al.*, 1990). However, H. Bäumlein *et al.* (personal communication) suggested that the short CATGCATG sequence motif within the conserved legumin box of the field bean legumin B4 gene is required for the legumin promoter activity, as well as its strict tissue specificity.

Sixty six nucleotides relative to the site of transcription initiation of the glycinin $A_{1a}B_{1b}$ subunit gene were required for embryo-specific expression, and a region from nucleotides −464 to −66 was required for correct quantitative expression levels (Goldberg *et al.*, 1989).

The DNA region −725 to −322 bp relative to the site of transcription initiation

of the sunflower helianthinin gene (*HaG3D* enhanced GUS expression in transgenic tobacco embryos (Jordano *et al.*, 1989). The A/T-rich sequence located from −705 to −654 nucleotides bound nuclear proteins (Jordano *et al.*, 1989).

Chimeric genes containing the 5′-flanking region of the type II glutenin gene and GUS reporter gene were introduced into tobacco by *A. tumefaciens*-mediated transformation (Takaiwa *et al.*, 1991). The region between positions −441 and −237 relative to the transcription initiation site was required for the temporal and endosperm-specific expression of GUS activity in tobacco (Takaiwa *et al.*, 1991). Nuclear proteins from immature seeds bound to the region between positions −130 and −120, and the interaction between the glutelin upstream region and the binding factors was highly sequence specific (Takaiwa and Oono, 1990). However, Kim and Wu (1990) obtained different results, in which the regions between positions −103 and −86, −122 and −108, −164 and −146, −206 and −189, and −595 and −575 were observed to interact with nuclear proteins, suggesting that the TGAGTCA motif is important for the interactions.

3. Zeins

Deletion mutants of the 19-kDa zein gene were constructed and transferred into sunflower using the Ti plasmid-derived vector (Roussell *et al.*, 1988). The DNA region between positions −337 and −125 relative to the site of transcription initiation was required for maximum transcription. Chimeric genes containing 5′-flaking regions and the GUS reporter gene were assayed by transient expression in carrot protoplasts (Thompson *et al.*, 1990). The DNA region between positions −114 and −8 exhibited low-level GUS activity. The sequence between positions −347 and −226 functioned as an enhancer in an orientation-independent manner; however, deletion of nucleotides −347 to −309 containing the prolamin box did not affect the enhancement, indicating that the prolamin box was not required for high-level expression (Thompson *et al.*, 1990).

Chimeric genes containing 5′-flanking regions of the 22-kDa zein gene and GUS reporter gene were introduced into tobacco using the Ti plasmid-derived vector (Schernthaner *et al.*, 1988; Matzke *et al.*, 1990). The region between positions −174 and +1 was sufficient for the temporal and endosperm-specific expression of GUS activity. The prolamin box is present at around position −300 of the 22-kDa zein gene and therefore does not seem to be required for specific expression. However, the sequence at around −130 bp upstream from the transcription initiation site, similar to that of the prolamin box, could be important (Matzke *et al.*, 1990). Twofold enhanced activity was observed with additional 5′ sequences up to −886 bp (Matzke *et al.*, 1990).

Quattrocchio *et al.* (1990) demonstrated by using the GUS reporter gene that each of two promoters of the 19-kDa zein gene showed temporal and endosperm-specific expression in *Petunia*. They further observed GUS activity in anthers of *Petunia* flowers.

Investigations of the interaction of the 5′-flanking sequence of the 19-kDa zein gene with nuclear proteins were carried out by Maier *et al.* (1987, 1988, 1990) and Grasser *et al.* (1990). They demonstrated that the region of −339 to −318 bp relative to the translation start site containing the prolamine box bound nuclear proteins (Maier *et al.*, 1987), and the A/T-rich region containing the CCAAT and TATA boxes bound nonhistone chromosomal high-mobility group proteins (Grasser *et al.*, 1990; Maier *et al.*, 1990).

Although the transcripts from the 22-, 19-, and 14-kDa zein genes were detected in transgenic cells or plants, the translational products were not detected (Matzke *et al.*, 1984; Goldsbrough *et al.*, 1986; Roussell *et al.*, 1988; Schernthaner *et al.*, 1988; Ueng *et al.*, 1988). This phenomenon is caused by ineffective translation and not by instability of the translational products in the transgenic cells or plants, because 22-kDa (Schernthaner *et al.*, 1988), 19-kDa (Schernthaner *et al.*, 1988; Williamson *et al.*, 1988), and 14-kDa (Hoffman *et al.*, 1987) zeins accumulated in tobacco plants and *Petunia* plants on using the phaseolin gene promoter for 14- and 19-kDa zeins or the 35S cauliflower mosic virus promoter for 19- and 22-kDa zeins instead of the zein gene promoter. However, Ohtani *et al.* (1991) observed that the newly synthesized 19-kDa zeins directed by the phaseolin gene promoter were degraded in tobacco seeds, having a half-life of less than 1 hr.

Several loci that affect zein synthesis have been identified. The recessive mutation at these loci reduces synthesis of some zeins (Kodrzycki *et al.*, 1989; Thompson and Larkins, 1989). The mutation at the *Opaque-2* locus causes a significant reduction of the 19-kDa zein and a dramatic reduction of the 22-kDa zein. Similar mutations were observed at *Opaque-6, Opaque-7,* and *Floury-2*. It has been suggested that these genes encode trans-acting factors that regulate zein gene expression (Thompson and Larkins, 1989). Recently, the cDNA and gene encoding the opaque-2 protein were isolated and sequenced (Hartings *et al.*, 1989; Maddaloni *et al.*, 1989; Schmidt *et al.*, 1990). The presumed amino acid sequences contain a domain similar to the leucine zipper motif, which is characteristic of some mammalian and fungal transcription activation factors. Schmidt *et al.* (1990) further demonstrated that the protein expressed from the cDNA in *E. coli* was bound to 5′-flanking sequence of the 22-kDa zein gene. The opaque-2 protein was present in the nucleus of maize seed (Varagona *et al.*, 1991). These results strongly suggest that the opaque-2 protein is a trans-acting factor. These are the only studies in which trans-acting factors regulating seed storage protein gene expression have been investigated in detail.

4. HMW Glutenin

The expression of a wheat genomic clone containing the entire coding sequence of HMW glutenin subunit 12 gene flanked by 2.6 kbp of 5′ sequences and 1.5

kbp of 3′ sequences was studied after introduction into tobacco using the Ti plasmid-derived vector (Robert *et al.*, 1989). It was concluded by using the CAT reporter gene that 433 bp upstream of the initiation codon was sufficient to confer temporal and endosperm-specific expression. Thomas and Flavell (1990) demonstrated by using the GUS reporter gene and the 35S promoter of cauliflower mosic virus that the region between −375 and −45 bp relative to the transcription start site was sufficient for the temporal and endosperm-specific expression in an orientation-independent manner. They concluded that the enhancer element for the tissue-specific expression was localized to a 40-bp region some 170 bp upstream of the transcription start site, because the fragment from −375 to −148 exhibited such a pattern of expression, but that from −375 to −186 did not (Thomas and Flavell, 1990).

The cis-control elements for the temporal and endosperm-specific expression of the HMW glutenin subunit Glu-1Dx5 gene was found in the region between −277 and +39 (Halford *et al.*, 1989).

C. PROCESSING, MODIFICATION, AND ASSEMBLY OF EXPRESSED PROTEINS IN TRANSGENIC PLANTS

The β-conglycinin α′ subunit accumulated in the transgenic *Petunia* seeds exhibited the expected size of 76 kDa, although several antigenically related polypeptides of about 68, 64, 60, and 55 kDa were detected (Beachy *et al.*, 1985). The α′ subunit and β subunit of β-conglycinin expressed in *Petunia* assembled into multimeric proteins with sedimentation coefficients of 7S–9S (Beachy *et al.*, 1985; Bray *et al.*, 1987). Phaseolin expressed in tobacco seeds was glycosylated and appeared to undergo removal of the signal peptide (Sengupta-Gopalan *et al.*, 1985) and was present in protein bodies (Greenwood and Chrispeels, 1985). However, a large proportion of the phaseolin was cleaved into discrete peptides (Sengupta-Gopalan *et al.*, 1985). Vicilin of pea was detected in protein bodies of transgenic tobacco seeds and some molecules were cleaved, as is the case in pea seed (Higgins *et al.*, 1988).

Pea legumin (Ellis *et al.*, 1988; Rerie *et al.*, 1991), field beam legumin (Bäumlein *et al.*, 1987), and helianthinin (Bogue *et al.*, 1990) expressed in transgenic tobacco were processed to form acidic and basic polypeptides, which are disulfide bonded. However, it has not been determined whether the processing site is the same as that in the original, and whether assembly into hexamers occurs. Helianthinin was present in protein bodies of tobacco seeds.

The 19-kDa zein accumulated in transgenic *Petunia* seeds exhibited a size corresponding to that of the mature protein, suggesting that cleavage of the signal peptide has occurred (Williamson *et al.*, 1988). The 14-kDa zein expressed in transgenic tobacco seeds was detected in vacuolar protein bodies (Hoffman *et al.*, 1987). The HMW glutenin subunit 12 expressed in transgenic tobacco seeds

displayed a solubility and molecular weight corresponding to the mature protein (Robert *et al.*, 1989).

Tobacco and *Petunia* are likely suited for examining the properties of the modified proteins, especially legume globulins, although further investigations on processing, modification, and assembly of the expressed proteins in transgenic plants are required.

X. FOOD PROTEIN ENGINEERING OF SEED STORAGE PROTEINS

The seed storage proteins synthesized on polyribosomes target the protein bodies and accumulate there in a highly packed state during seed maturation. These proteins remain stably in the protein bodies until germination is initiated. Once germination begins, the seed storage proteins are degraded and utilized as nitrogen and carbon sources. Targeting the protein bodies, forming the folded conformation that facilitates packaging in the protein bodies, stability, and degradation are likely to be governed by information borne in the primary and/or high order structures of the seed storage proteins. Moreover, functional properties depend on the structure of the seed storage proteins. Protein-engineered seed storage proteins should exhibit these properties. Otherwise, they cannot accumulate in a highly packed state in the protein bodies, be degraded at germination, or exhibit functional properties. In other words, any modification introduced into the seed storage proteins by protein engineering should not impair their natural properties. Experiments using transgenic plants are troublesome and time consuming. Therefore, it is desirable to evaluate precisely whether protein-engineered proteins are able to form proper conformations and to exhibit the expected functional properties, before the modified genes are introduced into the plants.

A. OBJECTIVES OF FOOD PROTEIN ENGINEERING

The relationships between structure and function of many enzymes have been studied in detail by means of protein engineering. Stabilization and alteration of reactions and substrate specificities of some enzymes have been achieved. These achievements are based on elucidation of the three-dimensional structure of these proteins by X-ray crystallography and computer graphics. Unfortunately, as already described, crystallization of seed storage proteins is difficult and rare, indicating that theoretically refined modifications, such as a single amino acid replacement by site-directed mutagenesis, to alter the properties of food proteins and to elucidate the relationship between functional properties and structure of food proteins, are restricted. However, structural characteristics, such as conserved and variable regions, and the relationships between structural and functional properties, such as gelation, emulsification, and breadmaking quality of seed storage proteins, make protein engineering possible. Objectives of food

TABLE XXXI

OBJECTIVES OF FOOD PROTEIN ENGINEERING

Functional properties
Elucidation of relationship between structure/conformation and specific functions
Improvement
Offering desired novel functions
Stability with regard to heat, pH, deteriorative reaction
Increasing or changing solubility
Nutritional value
Enrichment of limiting essential amino acids
Improvement of digestability
Physiological functions (biologically functional properties)
Enrichment
Improvement
Endowment
Creating cleavability
Novel proteins
Fusion with a protein that has different properties
Introduction of modification sites such as for glycosylation, phosphorylation, and lipophilization

protein engineering are listed in Table XXXI. More precise protein engineering may eventually be possible after some successful attempts in less refined protein engineering.

B. PROCEDURES IN FOOD PROTEIN ENGINEERING

The methods for achieving the objectives of food protein engineering are listed in Table XXXII. Random mutagenesis causes a mixture of random variants, where alterations are not based on theoretical modifications, and this demands a tedious selection of desired variants. The other methods can be carried out theoretically and then rationally pursued.

Oligonucleotide-mediated mutagenesis is frequently employed for protein engineering. In this method, substitution, insertion, and deletion of nucleotides are possible. The procedure of mismatch mutagenesis is illustrated in Fig. 14. Wild-type single-stranded recombinant DNA can be generated by using M13 or pUC118/119 vectors, which carry origins of replication (*ori*) derived from single-stranded bacteriophages (Sambrook *et al.*, 1989). Synthesized oligonucleotide primers carrying the desired mutation are annealled with the target single-stranded

TABLE XXXII

METHODS AVAILABLE FOR FOOD PROTEIN ENGINEERING

Method	Alteration
Site-directed mutagenesis	
Oligonucleotide-mediated mismatch mutagenesis	Nucleotide substitution, deletion insertion
Cassette mutagenesis	Nucleotide cluster subsitution, deletion, insertion
Insertion of gene fragment	Insertion
Enzymatic (Bal31, restriction enzymes) deletion	Deletion
Synthesis of genes	Arbitrary
Fusion of different genes	Fusion proteins
Random mutagenesis	
Chemical	Nucleotide substitution
Bisulfite G:C → A:T	
Nitrite G:C → A:T, A:T → G:C	
Methoxyamine G:C → A:T	
Exposure to ultraviolet light	Nucleotide substitution, deletion

DNA. The primer extension reaction is catalyzed by DNA polymerase using the single-stranded DNA as a template. The resultant double-stranded DNA is transformed into an appropriate *E. coli* strain. After transformation, double-stranded wild-type mutant DNAs are produced. For highly efficient screening of mutant DNA, several methods are employed: (1) the gapped duplex method (Kramer *et al.*, 1984), (2) the Kunkel method (Kunkel, 1985), and (3) the dNTP αS method (J. W. Taylor *et al.*, 1985). Mutagenesis kits based on these methods are commercially available. A single nucleotide mismatch and two or more mismatchs, respectively, require 8–9 nucleotides and 12–15 nucleotides on either side (Sambrook *et al.*, 1989).

Cassette mutagenesis requires restriction enzyme sites and synthesized double-stranded DNA. If the target DNA has appropriate restriction enzyme sites in the region that is to be modified, replacement of the DNA fragment with synthesized double-stranded DNAs that carry substitution, deletion, or insertion mutations is possible. If the target DNA has no appropriate restriction site, such sites can be introduced by mismatch mutagenesis. For the larger mutation, these methods are superior to the mismatch mutagenesis using a single-stranded DNA, because of the formation of a secondary structure in a long, single-stranded DNA. Insertion of gene fragments, enzymatic deletion, and fusion of different genes also

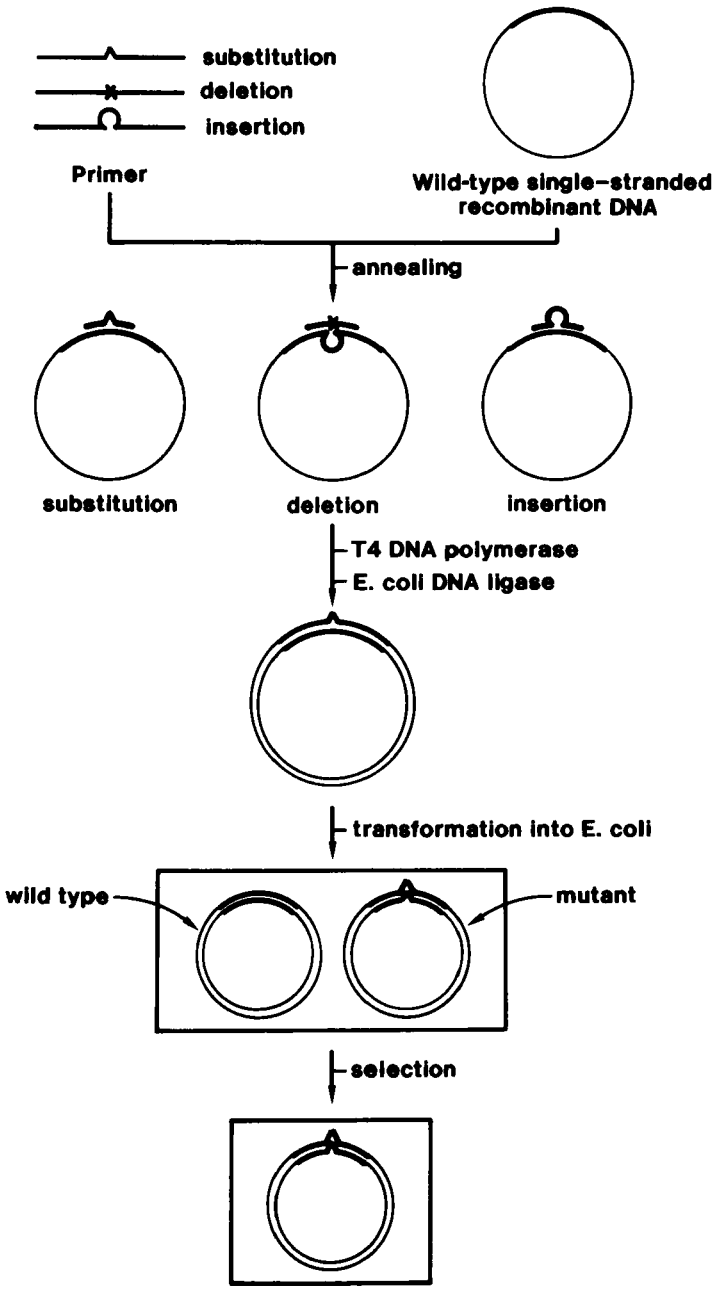


FIG. 14. Procedure for oligonucleotide-mediated mismatch mutagenesis. See text for details.

require a restriction enzyme site. In-frame constructions are necessary for getting mutant proteins by these methods.

C. SOYBEAN GLYCININ

When attempting to improve the nutritional value and functional properties of glycinin by protein engineering, the following problems should be considered: (1) Which regions of glycinin are susceptible to protein engineering? (2) What kinds of modifications can improve the nutritional value and functional properties? (3) What method of protein engineering can be conducted? (4) How are the results from the protein engineering to be evaluated?

1. Which Regions of Glycinin Are Susceptible to Protein Engineering?

Comparison of the amino acid sequences of various 11S globulins from legumes and nonlegumes revealed the variable and conserved regions as described in Section II,C,3 (see Table XVIII). According to the alignment by Wright (1988), the glycinin $A_{1a}B_{1b}$ subunit has five variable (I–V) and four conserved domains (Fig. 15A). Each variable domain exists in the hydrophilic region. On the other hand, Argos *et al.* (1985) suggested the presence of two variable domains, domain I and a hypervariable region, from the comparison of the amino acid sequence of 7S and 11S globulins (see Fig. 5). Domain I and the hypervariable region correspond, respectively, to the region from the variable domains I to II and the variable domain IV demonstrated by Wright (1988). The variable domains have little function in forming and maintaining the glycinin structure and may tolerate modification (Nielsen, 1985).

FIG. 15. (A) The variable and conserved domains of the glycinin $A_{1a}B_{1b}$ subunit aligned by Wright (1988). The number of the residues from the N-terminus for the variable domains (I–V) are shown above the alignment. Black areas are variable domains; open areas are conserved domains. Acidic and basic polypeptides are indicated. (B) Construction of the deleted proteins and $A_{1a}B_{1b}$-3. $A_{1a}B_{1b}$-3 lacks the N-terminal three amino acids, ΔI, the N-terminal 11; ΔII, from the 87th to the 113th; ΔIII, from the 161st to the 192nd; ΔIV, from the 244th to the 282nd, ΔV36, from the 441st to the C terminus; and ΔV8, from the 469th to the C terminus. The N-terminal methionine was retained in $A_{1a}B_{1b}$-3, ΔII, ΔIII, ΔV36, and ΔV8, and was cleaved in ΔI. ΔV36 and ΔV8 have two extra amino acids, Leu-Asn, at their C terminus. (C) Construction of the Met-inserted proteins. DI + 4Met and DI + 3Met have Arg-Met-Met-Met-Met and Arg-Gly-Met-Met-Met between Arg61 and Arg62, respectively. IV + 4Met has Arg-Met-Met-Met-Met-Gly between Pro281 and Arg282. V + 4Met has Glu-Met-Met-Met-Met-His between Pro467 and Gln468. (D) Construction of the disulfide bond-deleted proteins. Cys12 and Cys88 are substituted with Gly and Ser in Gly12 and Ser88, respectively. Both Cys12 and Cys88 afe, respectively, substituted with Gly and Ser in Gly12Ser88. [Partly from Kim *et al.* (1990b) and Utsumi and Kito (1991), with permission of the authors and publisher.]

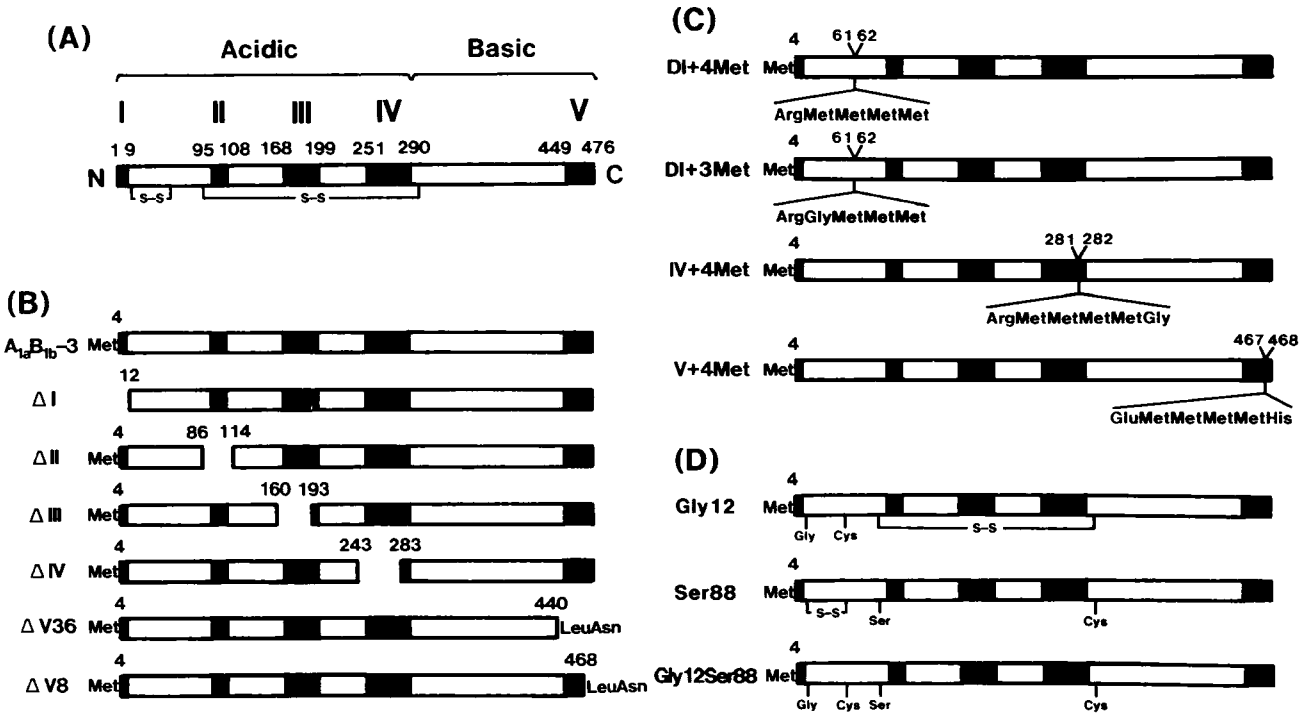

(A)
Acidic
Basic
I
II
III
IV
V
1 9
95 108 168 199 251 290
449 476
N
C
S–S
S–S
(B)
A1aB1b–3
Met
4
12
ΔI
ΔII
86 114
ΔIII
160 193
ΔIV
243 283
ΔV36
440
LeuAsn
ΔV8
468
LeuAsn
(C)
DI+4Met
6162
ArgMetMetMetMet
DI+3Met
6162
ArgGlyMetMetMet
IV+4Met
281 282
ArgMetMetMetMetGly
V+4Met
467 468
GluMetMetMetMetHis
(D)
Gly12
Gly
Cys
S–S
Ser88
S–S
Ser
Cys
Gly12Ser88
Gly
Cys
Ser
Cys

TABLE XXXIII

PROTEIN ENGINEERING TO IMPROVE FUNCTIONAL PROPERTIES OF GLYCININ

Functional properties	Modification	Effect
Gelation	Destabilization	Ease of gelation, increase in hardness
	Topology of Cys	Change in hardness and transparency
	Number of Cys	Change in transparency
Emulsification, foaming	Destabilization	Increase
	Increase of hydrophobicity	Increase

2. *What Kinds of Modifications Can Improve the Quality of Glycinin?*

Improvement of the nutritional properties of glycinin can be achieved by fortification of the limiting essential amino acid (methionine) and by improving its digestability.

Relationships between structural and functional properties of glycinin described in Section V,A and V,B answer the second question relating to the kind of protein engineering for improving the functional properties of glycinin (Table XXXIII).

Decreasing the heat stability of the glycinin molecule may reduce the heating time required for gelation, with a concomitant increase in gel hardness. Changing the topology of the free sulfhydryl residues in the glycinin molecule may increase the gel hardness and transparency. Decreasing the number of free sulfhydryl residues may increase the transparency of the gel.

Destabilization of the glycinin structure and increasing the hydrophobicity of the glycinin molecule may increase the emulsifying ability. Foaming is related to emulsification, thus foaming ability may be improved by similar modifications that improve emulsifying ability.

3. *How Can Protein Engineering Be Conducted?*

Protein engineering methods for improvement of glycinin quality involve exchange of domain, deletion or insertion of a certain region, insertion of synthetic DNA or a foreign gene fragment, and substitution of certain amino acids with other amino acids (Table XXXIV). Modifications that satisfy the first and the second problems mentioned previously should be conducted by these methods. The creation of novel glycinins with improved nutritional and/or functional properties is described below. The design strategy is based on the structural characteristics of glycinin and the putative relationships between structural and functional properties as described above.

TABLE XXXIV

METHODS OF PROTEIN ENGINEERING TO IMPROVE QUALITY OF GLYCININ

Method	Effect
Exchange of domain	Nutritional value, functional properties
Insertion of synthetic DNA or foreign gene fragment	Nutritional value, functional properties, physiological activities
Deletion of a certain region	Functional properties
Amino acid substitution	Nutritional value, functional properties, physiological activities

a. Exchange of Domains. Methionine concentrations are different among the constituent polypeptides of glycinin subunits (Table XXXV). The contribution of the constituent subunits to gelation also vary (Mori *et al.*, 1982b; Nakamura *et al.*, 1984a, 1985a). Exchange of the constituent polypeptides among the subunits to form artificial glycinin molecules is possible *in vitro* (Utsumi *et al.*, 1980a, 1983; Mori *et al.*, 1982b). Utsumi *et al.* (1987b) obtained an alternate $A_{1a}B_{1b}$ cDNA encoding four methionine residues in the DNA region corresponding to B_{1b} polypeptide. This indicates that exchange of the constituent polypeptides of the $A_{1a}B_{1b}$ mutant and A_2B_{1a} may enable construction of an artificial A_2B_{1b} subunit that can form the correct conformation, leading to an improvement in the nutritional value and functional properties. Such an exchange of the constituent polypeptides between subunits belonging to different groups (Table XXXV) may be possible based on the 45–49% absolute homology observed between the groups (Nielsen *et al.*, 1989).

b. Deletion of a Certain Region. All of the variable domains of glycinin subunits aligned by Wright (1988) are hydrophilic. Removal of each variable

TABLE XXXV

METHIONINE CONTENT IN GLYCININ SUBUNITS

		No. of Met residues[a]		
Group	Subunit	Acidic	Basic	Total
I	$A_{1a}B_{1b}$	3	3	6
	Mutant B_{1b}		4	
	$A_{1b}B_2$	3	2	5
	A_2B_{1a}	5	2	7
II	A_3B_4	2	2	4
	$A_5A_4B_3$	2	0	2

[a]From the references listed in Table XXI.

domain results in strengthening the hydrophobicity of the glycinin subunits as well as partial destabilization of the glycinin molecule. Consequently, the gelation and emulsification properties of glycinin could be improved.

Soybean proteins do not fully exhibit their functional properties in the acidic range (pH 3–6), wherein their solubility decreases due to their isoelectric points (at about pH 4.5). Soybean proteins are therefore rarely used for acidic foods such as mayonnaise and yogurt. Glycinin is expected to exhibit good solubility and functional properties in the acidic range after deleting the variable domains, which changes its net charge because the variable region contains many charged amino acids. In fact, phosphorylated soybean proteins exhibit good emulsifying activity and gel-forming ability (Hirotsuka *et al.*, 1984).

Expression plasmids for ΔI, ΔII, ΔIII, ΔIV, ΔV36, and ΔV8 (Fig. 15B) from $pKGA_{1a}B_{1b}$-3 have been constructed (Kim *et al.*, 1990b).

c. Insertion of Synthetic DNA or Foreign Gene Fragments. Insertion of synthetic DNA or foreign gene fragments encoding plural methionine into DNA regions corresponding to the variable domains improves the nutritional value of glycinin. Insertion of synthetic DNA or foreign gene fragments encoding physiologically active peptides flanked by protease cleavage sites into DNA regions corresponding to the variable domains endows glycinin with physiological activity that is useful for maintenance and promotion of human health. It is known that dipeptides and tripeptides can be absorbed in the human alimentary canal (Adibi and Phillips, 1968; Craft *et al.*, 1968). Some dipeptides and tripeptides with physiological activities have been observed (Table XXXVI). An alteration derived from insertion of DNA encoding such a small peptide into a DNA region corresponding to the variable region would not disturb the formation of the correct conformation of the modified glycinin. If the hydrophilicity of the variable domains is partly disturbed by such an insertion, glycinin molecules are partly destabilized, possibly resulting in the improvement of gel-forming and emulsifying abilities. However, excessive destabilization is not desirable.

Kim *et al.* (1990b) constructed expression plasmids for DI + 4Met, DI + 3Met, IV + 4Met, and V + 4Met (Fig. 15C) from $pKGA_{1a}B_{1b}$-3 using rare restriction enzyme sites. As a result of the insertion of peptide, the secondary structure in the vicinity of the insertion site, predicted according to the procedure of Chou and Fasman (1974a,b), changed from random to α helix for DI + 4Met, β turn to α helix for IV + 4Met, and random to α helix for V + 4Met; there apparently was no change for DI + 3Met.

d. Oligonucleotide-Mediated Mutagenesis. Single or several amino acids can be replaced by other amino acids and can also be deleted or inserted using oligonucleotide-mediated site-directed mutagenesis to improve nutritional value, functional properties, and physiological activities.

If the target proteins contain physiologically active peptide sequences or similar

TABLE XXXVI
DI- AND TRIPEPTIDES WITH PHYSIOLOGICAL ACTIVITY

Peptide	Physiological activity	Reference
Dipeptides		
Tyr–Arg	Morphinelike	Takagi *et al.* (1979)
Val–Trp	Inhibits angiotensin-converting enzme	Cheung *et al.* (1980)
Ile–Trp	Inhibits angiotensin-converting enzyme	Cheung *et al.* (1980)
Ile–Tyr	Inhibits angiotensin-converting enzyme	Cheung *et al.* (1980)
Tripeptides		
Gly–His–Lys	Liver cell growth factor	Pickart *et al.* (1973)
Gly–Gly–His	Cu binding	Lau *et al.* (1974)
Gly–Pro–Arg	Inhibits fibrin polymerization	Laudano and Doolittle *et al.* (1978)
Ile–Pro–Ile	Inhibits dipeptidyl aminopeptidase IV	Umezawa *et al.* (1984)
Val–Pro–Leu	Inhibits dipeptidyl aminopeptidase IV	Umezawa *et al.* (1984)
Leu–Val–Leu	Inhibits angiotensin-converting enzyme	Hazato and Kase (1986)

amino acid sequences, alterations of the amino acid sequences to more desirable sequences and cleavage by digestive enzymes are possible. Also, some contiguous amino acids in the variable domains can be replaced with physiologically active peptides according to this method.

The glycinin $A_{1a}B_{1b}$ subunit has a polyglutamate region in the variable domain IV. Deleting this region or substituting this region with other amino acids, such as polyglutamine (no charge, but hydrophilic) or polylysine (positively charged), could change the net charge of the glycinin molecule without disturbing the folding. Sulfhydryl residues and disulfide bonds are closely related to the gel-forming ability, as described in Section V,A. Therefore, introduction or deletion of sulfhydryl residues and disulfide bonds may improve the gel-forming ability of glycinin. Deletion of disulfide bonds may cause destabilization of the molecule, resulting in an improvement in emulsifying ability.

Expression plasmids for disulfide bond-deleted glycinins (Fig. 15D) and IV(ΔGlu), IV(Gln), and IV(Lys) from $pKGA_{1a}B_b$-3 have been constructed (S. Utsumi *et al.*, unpublished observations).

4. *Evaluation of Abilities of Modified Glycinins to Form the Correct Conformation*

To achieve improved glycinin qualities by means of protein engineering, the modified proteins should be able to assume the correct conformation. Thus, how to judge the formation of the correct conformation is a critical question.

Kim *et al.* (1990a) demonstrated that *E. coli* strain JM105 accumulated ex-

pressed, unmodified proteins from $pKGA_{1a}B_{1b}$-3 at the level of 20% of the total bacterial proteins, and that the expressed protein was soluble and self-assembled into trimers with a secondary structure similar to that of soybean glycinin. Moreover, it has been suggested that the folding of the expressed proteins with the signal peptide could be disturbed on account of the strong hydrophobicity of the signal peptide, which renders the protein susceptible to proteinase digestion (Utsumi *et al.*, 1987a, 1988a). Accordingly, the following criteria were assessed for the formation of the correct conformation: (1) high-level expression, (2) solubility, and (3) self-assembly into trimers. These three criteria were applied to modified proteins expressed in JM105 cells harboring individual expression plasmids (Table XXXVII) (Kim *et al.*, 1990b; S. Utsumi *et al.*, unpublished observations). Among the modified proteins, ΔI, ΔV8, IV + 4Met, V + 4Met, Gly12, Ser88, Gly12Ser88, IV(ΔGlu), IV(Gln), and IV(Lys) accumulated as soluble proteins at higher levels in cells and self-assembled into trimers. It was then concluded that these 10 modified proteins could self-assemble into a conformation similar to that of the native glycinin, that the disulfide bonds of Cys12–Cys45 and Cys88–Cys298 are not necessary to form a correct conformation, and that it is possible to dramatically change the net charge of the glycinin molecule.

TABLE XXXVII

EXPRESSION LEVELS OF MODIFIED PROGLYCININ $A_{1a}B_{1b}$ cDNAs IN *E. COLI* AND PROPERTIES OF EXPRESSED PROTEINS

Name of modified protein	Method of modification	Level of expression[a]	Solubility	Assembly
ΔI	Deletion	3	Yes	Yes
ΔII	Deletion	1	No	?[b]
ΔIII	Deletion	1	No	?
ΔIV	Deletion	2	No	?
ΔV36	Deletion	2	No	?
ΔV8	Deletion	3	Yes	Yes
DI + 4Met	Met insertion	1	No	?
DI + 3Met	Met insertion	0	—[c]	—
IV + 4Met	Met insertion	3	Yes	Yes
V + 4Met	Met insertion	2	Yes	Yes
Gly12	Substitution	3	Yes	Yes
Ser88	Substitution	3	Yes	Yes
Gly12Ser88	Substitution	3	Yes	Yes
IV(ΔGlu)	Deletion	3	Yes	Yes
IV(Glu)	Substitution	3	Yes	Yes
IV(Lys)	Substitution	3	Yes	Yes

[a]O, No expression; 1, <0.1%; 2, 5–10%; 3, 10–20%.

[b]Unknown.

[c]Experiment not possible.

Since, except for ΔI, the constructs of individual expression plasmids in the vicinity of the translation initiation site and the promoter are identical, the efficiency of translation and transcription may be identical. However, the accumulated amounts of ΔII, ΔIII, DI + 4Met and DI + 3Met in the cells were much smaller than those of the other modified proteins (Table XXXVII). Therefore, it is possible that the modifications introduced into ΔII, ΔIII, DI + 4Met, and DI + 3Met may have disturbed the correct folding, making them susceptible to proteinase digestion. This suggests that domain I specified by Argos *et al.* (1985) may not tolerate modification. In fact, a modified $A_{1a}B_{1b}$ protein that lacks 31 of the N-terminal amino acids did not accumulate in the cells (Kim *et al.*, 1990b). However, it is difficult to conclude that the variable domains II and III are not targets for modification, because ΔII and ΔIII lack part of the conserved domains (Fig. 15B). On the other hand, ΔIV and ΔV36 accumulated in the cells in much larger amounts than did ΔII and ΔIII, although ΔII, ΔIII, ΔIV, and ΔV36 were all insoluble. This, together with the fact that IV + 4Met is soluble, suggests that deletion of the hydrophilic variable domains IV and V did not disturb the folding of ΔIV and ΔV36, but made them insoluble after folding.

5. *Functional Properties of Modified Glycinins*

Four (ΔI, ΔV8, IV + 4Met, and V + 4Met) of the 10 modified proteins that were established to fold into the proper conformation were purified from *E. coli* strain JM105 to homogeneity by salt precipitation, Q-Sepharose column chromatography, and cryoprecipitation (Kim *et al.*, 1990b). The function properties (gelation and emulsification) of the purified, modified proteins were compared with those of native glycinin. Table XXXVIII shows the emulsifying activity of the native and the modified glycinins. Each of the modified proteins, especially

TABLE XXXVIII

EMULSIFYING ACTIVITY OF MODIFIED GLYCININS[a]

Sample	Emulsifying activity[b] (%)
Soybean glycinin	100
$A_{1a}B_{1b}$-3	130
ΔI	132
ΔV8	215
IV + 4Met	134
V + 4Met	211

[a]Data from Kim *et al.* (1990b), with permission of the authors and publisher.

[b]Emulsifying activity was expressed as relative value (%) compared with the soybean glycinin.

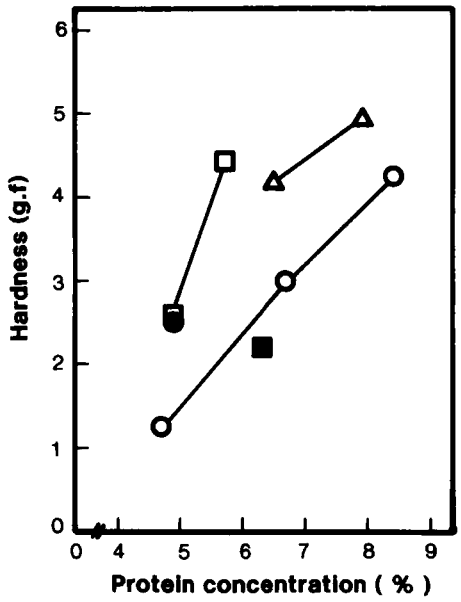


FIG. 16. Hardness of gels from modified proteins. Protein samples in 3.5 m*M* K–P_i buffer (pH 7.6) were boiled for 30 min. ○, Native glycinin; □, ΔI; △, IV + 4Met; ●, V + 4Met; ■, ΔV8. (From Kim *et al.*, 1990b, with permission of the authors and publisher.)

ΔV8 and V + 4Met, exhibited higher values than the native glycinin. This suggests that the hydrophobicity of the C-terminal region may be closely related to the emulsifying properties of glycinin. All of the modified proteins examined here also formed gels following heating at 100°C. The hardness of the gels from the native and modified glycinins is shown in Fig. 16. The gels from ΔI, IV + 4Met, and V + 4Met exhibited a higher degree of hardness than did the native glycinin gel, whereas ΔV8 was slightly softer. However, at present we cannot explain the differences in hardness among the modified proteins. Finite modifications are required to explain such phenomena.

S. Utsumi *et al.* (unpublished observations) observed excellent gel-forming ability in Ser88. This indicates that changes in the number and/or the topology of free sulfhydryl residues of glycinin are powerful methods for improving the gel-forming ability.

D. FIELD BEAN LEGUMIN

Subunits of legumin of *V. faba* are classified into two groups, A and B (Wobus *et al.*, 1986). Group B subunits are completely free of methionine, and group A subunits contain up to four methionine residues per subunit (Saalbach *et al.*, 1988).

Saalbach *et al.* (1988) introduced four methionine codons into the legumin B gene by changing the reading frame near the 3′ end and substituting the stop codon (TAA), which was introduced by the frame shift, with ATG (methionine) during oligonucleotide-mediated site-directed mutagenesis. They also constructed a hybrid gene containing 10 methionine codons from a soybean glycinin A_2B_{1a} gene and the modified legumin gene. The hybrid gene encodes a hybrid composed of the acidic polypeptide of A_2B_{1a}, the N-terminal one-third of B_{1a}, and the C-terminal two-thirds of the modified legumin basic polypeptide. The modified genes were transformed into tobacco using an *A. tumefaciens* Ti plasmid. Although Northern blots revealed the expression of mRNA having the expected size, the expressed proteins could not be detected. The modified proteins have more hydrophobic properties at C-terminal regions than does the native one. Saalbach *et al.* (1990) suggested that the alteration of the characteristic structure caused by the modification interfered with intracellular transport. In fact, Saalbach *et al.* (1991) observed that the C-terminal 76 residues included the vacuolar targeting signal. However, this does not exclude the possibility that the structural alteration induced by the modification disturbed the folding of the expressed protein.

E. FRENCH BEAN PHASEOLIN

Phaseolin β subunit contains only three methionine residues. For improving the nutritional value of phaseolin, Hoffman *et al.* (1988) constructed a modified β-phaseolin gene by the insertion of a 45-bp synthetic DNA encoding a methionine-rich region of 14-kDa zein. The predicted secondary structure of the inserted peptide region is an α helix, matching the structure of the peptide surrounding the insertion site. The modified gene was introduced into tobacco through *A. tumefaciens*-mediated transformation. The modified gene was expressed at a level similar to that of the normal gene at the mRNA level, but the quantity of the modified phaseolin was consistently much lower than normal. It was concluded that the modified phaseolin was transported through endoplasmic reticulum and the golgi apparatus and was then degraded either in the golgi apparatus or in protein bodies. The insertion site of phaseolin corresponds to a homology region B in the conserved domain II aligned by Wright (1988). This indicates that the region surrounding the insertion site is important for the formation of the correct conformation. This was confirmed later by X-ray crystallography of phaseolin (Lawrence *et al.*, 1990).

F. MAIZE ZEIN

Zeins are deficient in lysine and tryptophan. Wallace *et al.* (1988) constructed modified 19-kDa zein genes by site-directed mutagenesis to substitute lysine residues for neutral amino acids and by insertion of synthetic DNAs to increase

the methionine and tryptophan codons. The modified sites were in the antiparallel helices, the turn regions between the antiparallel helices, and the N-terminal region (see Fig. 2). Messenger RNAs for the modified zeins were synthesized *in vitro* by using the SP6 system and were injected into *Xenopus laevis* oocytes. The modifications did not affect the translation of the modified genes, signal peptide cleavage, or stability of the modified zeins.The expressed zeins self-aggregated into dense structures in the oocytes.

Chimeric genes composed of the β-phaseolin promoter, a 19-kDa zein coding sequence and its modified versions, and a β-zein polyadenylation signal were introduced into tobacco by *A. tumefaciens*-mediated transformation (Ohtani *et al.*, 1991). However, newly synthesized 19-kDa zeins as well as modified zeins were degraded in tobacco seeds within a short time. Hoffman *et al.* (1987) observed the accumulation of 15-kDa (14-kDa) zeins in tobacco seeds at a level of 1–1.5% of the total proteins. In relation to these observations, Ohtani *et al.* (1991) suggested that the structural differences between 19- and 15-kDa zeins could account for their differences in processing and stability in the seeds of transgenic tobacco. In this case, the modified zeins could also accumulate in maize seeds.

G. WHEAT PROTEINS

As described in Section V,C, HMW glutenin subunits mainly contribute to breadmaking quality based on the structural characteristics that enable formation of elastic β-spiral structures; these structures have sulfhydryl residues at the N- and C-terminal regions to form head-to-tail polymers (Shewry and Tatham, 1990). Some glutenin subunits (e.g., 12) are associated with poor breadmaking quality. Such subunits have less regularity of repetitive β turns than do those associated with good breadmaking quality; this relates to properties of repeating hexamer and nonamer units of the consensus type (Flavell *et al.*, 1989; Goldsbrough *et al.*, 1989). These data indicate that substitution of amino acids in repeat units to make repeat units close to the consensus sequences may enhance the contribution of HMW glutenin subunits to breadmaking quality.

The ω-gliadin is presumed to form β-spiral structures, but it cannot form an elastic polymer because it contains no cysteine residue (Tatham *et al.*, 1985). Thus, introduction of cysteine residues into the N- and C-terminal regions of ω-gliadin may improve the breadmaking quality of wheat proteins.

LMW glutenin subunits are presumed to contribute to pasta cooking quality due to their tendency to form aggregates through disulfide bonds (Feillet *et al.*, 1989). However, α/β- and γ-gliadins do not contribute to pasta cooking quality, because they prefer to be monomers. α/β- and γ-gliadins and LMW glutenin subunits share structural characteristics, thus it may be possible to use α/β- and γ-gliadins to contribute to pasta cooking quality by introducing sulfhydryl residues that react to form disulfide bonds.

H. RICE PROTEINS

The predominant storage proteins of rice seeds are prolamins and glutelins, which are packaged in protein bodies I and II, respectively (see Section III,A). PB-I is barely digestable because is has a concentric ring structure.

The amino acid sequences of the signal peptides of various seed storage proteins were compared and the seed storage proteins were classified accordingly, depending on their accumulation types (direct and indirect accumulation; see Section III,A). Thus, the signal peptides may play an essential role in the distribution of the storage proteins (Tanaka and Masumura, 1988; Masumura *et al.*, 1990). These facts suggest that the exchange of the signal peptide of glutelin with that of prolamin may make glutelin accumulate in PB-I, thereby making PB-I digestable (Tanaka and Masamura, 1988).

XI. CONCLUSIONS AND RESEARCH NEEDS

Recent advances in the elucidation of the structural characteristics, the relationships between the structural and functional properties of seed storage proteins, and the techniques for gene manipulation have made possible the use of protein engineering in seed storage proteins to improve their food-related qualities. Developments in techniques for the generation of transgenic plants have made possible the elucidation of the regulation mechanisms of seed storage protein gene expression and also of transgenic plants harboring modified genes. However, properties of modified proteins should be examined before the modified genes are transferred into plants. This is supported by failed attempts to produce modified proteins in transgenic plants.

Transgenic crop breeding that produces novel seed storage proteins with high food qualities is now becoming possible. However, to design novel proteins theoretically and to achieve transgenic crop breeding, the following studies are needed.

1. Elucidation of the higher order structure of seed storage proteins by X-ray crystallography and computer graphics.
2. Clarification by protein engineering of the mechanisms of the functional properties at the level of the primary and higher order structures of seed storage proteins.
3. Elucidation by protein engineering of the relationships between the functional properties and the primary and the higher order structures of seed storage proteins.
4. Elucidation of the mechanisms of expression of seed storage protein genes, including trans-acting factors and their gene structure.

5. Establishment of easy and efficient methods for transformation, including gene targeting and regeneration of important crops.

6. Achievement of specific and high-level expression of modified genes in seeds of transgenic crops.

7. Evaluation of the safety of transgenic crops.

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ADVANCES IN FOOD AND NUTRITION RESEARCH, VOL. 36

MINERALS AND TRACE ELEMENTS IN MILK

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I. INTRODUCTION

Minerals and trace elements occur in the body in a number of chemical forms, such as inorganic ions and salts, or as constituents of organic molecules, for example, proteins, fats, carbohydrates, and nucleic acids. They serve a wide variety of essential physiological functions, ranging from structural components

of body tissues to essential components of many enzymes and other biologically important molecules.

This review outlines the nutritional roles, recommended intakes, and hazards of deficiency or excess of the 20 minerals and trace elements that are considered to be nutritionally essential for man, all of which occur in both human and cow milk. The content, chemical form, bioavailability, and nutritional significance of these minerals in both human and cow milk are considered together with comparative aspects of human and cow milks. In addition, in view of the widespread use of infant formulas based on cow milk, some nutritional aspects of minerals in these formulas are discussed.

The 20 minerals that are considered essential in the human diet are sodium, potassium, chloride, calcium, magnesium, phosphorus, iron, copper, zinc, manganese, selenium, iodine, chromium, cobalt, molybdenum, fluoride, arsenic, nickel, silicon, and boron. While some of these, e.g., arsenic, nickel, silicon, and boron, have not been shown to be essential for humans, they are essential for experimental animals and probably are also essential for man. A number of other chemical elements occur in milk, e.g., lithium, boron, bromine, aluminum, strontium, silver, lead, tin, vanadium, mercury, cadmium, rubidium, and cesium. These are not nutritionally essential and are not reviewed here, but many of them are toxic. However, their concentrations in milk are normally well below toxic levels, and these have been reviewed by others (Jarrett, 1979; Murthy, 1974; Lee and Lorenz, 1979).

The essential minerals are sometimes classified into two groups, i.e., the macrominerals and trace elements. The macrominerals (sodium, potassium, chloride, calcium, magnesium, and phosphorus) are present in the body in amounts greater than about 0.01% by weight, while the trace elements (the remaining 14 essential minerals) occur in the body at much lower levels and are required in the diet in amounts less than about 100 mg/day (Briggs and Calloway, 1979). Many of the essential minerals, particularly the trace elements, are toxic when ingested in excess of requirements. However, the levels at which they exert their toxic effects are generally well in excess of their normal levels in milk and other foods, and for this reason their toxicological aspects will only be discussed where they are considered relevant.

There is much less information on the nutritional aspects of some minerals than others, and a considerable amount of current research is being carried out to clarify the roles of minerals in nutrition. This review includes some of the more recent findings in relation to the nutritional significance of minerals, particularly calcium and trace elements, in milk.

Although the minerals are treated separately, it is important to realize that interactions of minerals with each other, with other constituents of milk, and with other food constituents occur, and such interactions are assuming an increasing importance in nutrition.

II. MINERAL AND TRACE ELEMENT CONTENT OF BOTH HUMAN AND COW MILK

The mineral and trace element content of human and cow milks is not constant but is influenced by a number of factors, such as stage of lactation, nutritional status of the mother, and environmental and genetic factors. Reported values in the literature for the concentration of many minerals and trace elements show a wide variation, which is partly due to these factors, but is also partly due to analytical errors and contamination from milk collection and milk-processing equipment and procedures. Representative values for the average mineral content of mature human and cow's milks are presented in Table I.

The total mineral content of cow milk (~7.3 g/liter) is considerably higher than that of mature human milk (~2.0 g/liter) (Renner, 1983). This is mainly

TABLE I

MEAN CONCENTRATIONS OF MINERALS AND TRACE ELEMENTS IN MATURE HUMAN MILK AND COW MILK[a]

Component[b]	Mature human milk	Reference	Cow milk	Reference
Sodium (mg)	180	AAP (1985)	500	Paul and Southgate (1978)
Potassium (mg)	525	AAP (1985)	1500	Paul and Southgate (1978)
Chloride (mg)	420	AAP (1985)	950	Paul and Southgate (1978)
Calcium (mg)	280	AAP (1985)	1200	Paul and Southgate (1978)
Phosphorus (mg)	140	AAP (1985)	950	Paul and Southgate (1978)
Magnesium (mg)	35	AAP (1985)	120	Paul and Southgate (1978)
Iron (mg)	0.3	AAP (1985)	0.5	Paul and Southgate (1978)
Zinc (mg)	1.2	AAP (1985)	3.5	Paul and Southgate (1978)
Copper (mg)	0.25	AAP (1985)	0.09	Pennington *et al.* (1987)
Manganese (μg)	6	AAP (1985)	30	Lonnerdal *et al.* (1981)
Iodine (μg)*	64–178	Pennington, 1988	100–770	Pennington (1988)
Fluoride (μg)	16	AAP (1985)	20	Taves (1983)
Selenium (μg)*	16	Smith *et al.* (1982)	10	Pennington *et al.* (1987)
Cobalt (μg)	0.1	Smith (1987)	0.5	Smith (1987)
Chromium (μg)	0.27	Casey *et al.* (1985)	2	Muzzarelli *et al.* (1983)
Molybdenum (μg)	2	Casey and Neville (1987)	50	Tsongas *et al.* (1980)
Nickel (μg)	1.2	Casey and Neville (1987)	26	Kirchgessner *et al.* (1967)
Arsenic (μg)	0.2–0.6	Anke (1986)	20–60	Anke (1986)
Silicon (μg)	700	Renner (1983)	3000	Nielsen (1988)
Boron (μg)	60–80	Nielsen (1986)	500–1000	Nielsen (1986)

[a]Concentration given as content per liter.

[b]Asterisks denote minerals or elements whose concentrations are strongly influenced by dietary intake.

due to the higher concentrations of sodium, potassium, chloride, calcium, phosphorus, and magnesium in cow milk (Table I).

III. SODIUM, POTASSIUM, AND CHLORIDE

Sodium is the principal cation of extracellular fluid and is the primary regulator of extracellular fluid volume. It is important in the regulation of osmolarity, acid–base balance, and the membrane potential of cells, as well as in active transport across cell membranes. Chloride is the principal extracellular anion and is essential in the maintenance of fluid and electrolyte balance. Potassium is the principal intracellular cation, occurring in the cell at a concentration (145 mEq/liter) more than 30 times its concentration in extracellular fluid. Extracellular potassium contributes to the transmission of nerve impulses, to the control of skeletal muscle contraction, and to the maintenance of blood pressure (NRC, 1989a).

Under normal circumstances, dietary deficiency of sodium, potassium, or chloride does not occur, but the body can be depleted of sodium and chloride under extreme conditions, e.g., heavy perspiration, chronic diarrhea, or renal disease. Body depletion of potassium can occur in conditions in which there are large alimentary or renal losses (NRC, 1989a).

Minimum requirements for sodium, potassium, and chloride (e.g., 500, 2000, and 750 mg/day, respectively, in adults) have been established (NRC, 1989a). Actual intakes of sodium and chloride are considerably greater than minimum requirements in many populations and, given the evidence of a relationship of high salt intake to hypertension, it has been recommended that sodium intake be limited to 2.4 g/day (NRC, 1989b). Similarly, considering the possible beneficial effect of potassium in hypertension, increasing potassium intake beyond the minimum requirement has been recommended (NRC, 1989b).

Cow milk contributes little to dietary intakes of sodium (7% in the United Kingdom; Hazell, 1985), but some dairy products, such as cheese and butter, contain added salt and can be significant sources of sodium in some countries (e.g., about 13% of total sodium intake in the United Kingdom; Hazell, 1985). It has been estimated that milk and dairy products provide 20% of total sodium and 24–29% of total potassium in the diet in Ireland and the United Kingdom (Flynn *et al.*, 1990).

Mature human milk contains considerably less sodium, potassium, and chloride than cow milk (Table I). The concentrations of sodium, potassium, and chloride have been reported to decrease from 480, 740, and 850 mg/liter, respectively, in colostrum to 160, 530, and 400 mg/liter, respectively, in mature human milk (Blanc, 1981). No relationship has been demonstrated between maternal dietary sodium, potassium, or chloride intakes and the concentrations of these electrolytes in milk (Ereman *et al.*, 1987; Lonnerdal, 1986).

Particularly high levels of sodium occur in cow colostrum (Kirchgessner *et al.*, 1967), but these decrease within a few days to the levels shown in Table I. The sodium content of cow milk is not influenced by dietary sodium intake within the normal range (Kirchgessner *et al.*, 1967). Sodium concentration in milk tends to be higher at the end of lactation when milk yields are low (Kirchgessner *et al.*, 1967). In contrast to most other minerals, the potassium concentration in cow colostrum is lower than that of mature milk, but increases to normal values within the first 2–3 days of lactation and is independent of potassium intake (Kirchgessner *et al.*, 1967). The chloride concentration in cow milk decreases from colostrum to mature milk but increases sharply toward the end of lactation and is independent of dietary intake (Kirchgessner *et al.*, 1967).

Sodium, potassium, and chloride are believed to be present in milk almost entirely as free ions (Holt, 1985). Practically all the sodium, potassium, and chloride in milk is absorbed in the gastrointestinal tract, although much of what is absorbed is not retained (DHSS, 1980).

The concentrations of sodium, potassium, and chloride in milk are of physiological importance in the feeding of the young infant, and clinical problems may arise if there is an excessive intake of these nutrients. The kidney of the young infant, compared to that of the adult, has a limited capacity to concentrate solids, and the renal solute load exerts a major effect on water balance. Renal solute load is determined mainly by sodium, potassium, chloride, phosphorus, and protein (which gives rise to urea). Cow milk has a much higher potential renal solute load (~300 mOsmol/liter) than human milk (~93 mOsmol/liter) (Ziegler and Fomon, 1989). The high renal solute load resulting from ingestion of cow milk may be of relatively little significance in most circumstances because the kidney merely excretes a more concentrated urine. However, it does lead to a smaller margin of safety against dehydration, which can occur in conditions of diarrhea, fever, and low water intake, and for this reason it is recommended that the upper limit of potential renal solute load in formulas for young infants should be about 220 mOsmol/liter (Ziegler and Fomon, 1989). Recommended concentrations of sodium, potassium, and chloride in infant formulas are in the ranges 150–350, 500–1000, and 400–800 mg/liter, respectively (DHSS, 1980), and 133–403, 534–1338, and 369–1360 mg/liter, respectively (AAP, 1985). Cow milk based infant formulas currently in use fall comfortably within these guidelines (Ziegler and Fomon, 1989).

IV. CALCIUM

The adult human body contains about 1200 g of calcium, which amounts to about 1.5–2% of body weight. Of this, 99% is found in bones and teeth, where it is present as calcium phosphate, providing strength and structure. The remaining 1%, found in extracellular fluids and intracellular structures and cell

membranes, is responsible for a number of regulatory functions, such as maintenance of normal heart beat, blood coagulation, hormone secretion, integrity of intracellular cement substances and membranes, nerve conduction, muscle contraction, and activation of enzymes (NRC, 1989a; British Nutrition Foundation, 1989).

Cows' milk and cow milk products, such as cheese and yogurt, are very good sources of dietary calcium (Miller, 1989). The contribution of dairy products to total calcium intake has been estimated as 75% in The Netherlands (Schaafsma, 1984), from 55% (dietary intake data; Block *et al.*, 1985) to 72% (food disappearance data; Marston and Welsh, 1984) in the United States, 60% in the United Kingdom (Hazell, 1985), and 52% in Ireland (INDI, 1990).

In the absence of dietary milk and dairy products, calcium intakes in excess of 300 mg/day are difficult to achieve (Schaafsma, 1984). This is far below the recommended dietary allowance (RDA) for calcium in the United States, which has been set at 800 mg for adults and children aged 1–10 years and 1200 mg for adolescents, and young adults, and pregnant and lactating women (NRC, 1989a). On this basis, it might be considered that consumption of dairy products is very important in order to achieve an adequate calcium intake. However, there is still considerable disagreement on human calcium requirements, and this is reflected in the wide variation in adult RDAs for calcium (400–1200 mg) that have been set by different authorities (International Union of Nutritional Sciences, 1983).

The calcium content of human milk is considerably lower than that of cow milk (Table I). Harzer *et al.* (1986a) reported that the calcium concentration in human milk increases from a mean of 250 mg/liter at day 1 to 320 mg/liter at day 5 and remains constant at about 300 mg/liter up to day 36 of lactation. Vaughan *et al.* (1979) reported that the calcium concentration in human milk decreases by 30% between the first and ninth months postpartum. There appears to be no correlation between the intake of calcium and the calcium concentration in milk (Vaughan *et al.*, 1979; Kirskey *et al.*, 1979). In cow milk, calcium concentration is slightly elevated in colostrum and at the end of lactation but varies little with feeding or season (Renner, 1983; Holt, 1985).

In cow milk, 99% of the calcium is in the skim milk fraction (Fransson and Lonnerdal, 1983). Two-thirds of the total calcium occurs in colloidal form associated with the casein micelles, either in a calcium phosphate salt (about half of total milk calcium) or as calcium ions bound to phosphoserine residues (about one-sixth of total calcium); the remaining one-third is soluble (Holt, 1985). Ionized calcium in the soluble phase accounts for about 10% of the total calcium (Jenness, 1974) and most of the remaining soluble calcium occurs as calcium citrate (Holt, 1985). A small amount of calcium (0.15%) is bound to α-lactalbumin (Lonnerdal and Glazier, 1985).

In human milk, 16–26% of calcium is present in the lipid fraction (bound to

the fat globule membrane), 35% is protein bound (6% to casein and the remainder to whey proteins), and about 40% is soluble, mainly as calcium ion (Allen and Neville, 1983; Harzer *et al.*, 1986b; Fransson and Lonnerdal, 1983). About 1% of milk calcium is bound to α-lactalbumin (Lonnerdal and Glazier, 1985).

In recent years, considerable attention has been focused on the bioavailability of calcium (and trace elements) in milk. There is no clear, widely accepted definition of bioavailability as it applies to dietary minerals and trace elements. A confounding factor is the physiological need of the animal for the nutrient, which usually results in an inverse relationship between fractional absorption and physiological status and a more efficient absorption of small amounts. Welch and House (1984) have proposed a definition that is presumably independent of the physiological status of the animal: "Bioavailability to organisms of a mineral element is that proportion of an element in a nutrient medium which is potentially absorbable in a form which is metabolically active." In practice this is difficult to measure, since it is not usually possible to measure absorption in the absence of status as a confounding factor. This difficulty has been overcome for iron, by correcting observed absorption values using the absorption of a standard reference dose in the same subject (Hallberg, 1981). This gives a working definition of bioavailability: "The efficiency of utilization of a nutrient in a food or diet relative to an appropriate standard" (Miller, 1989). However, this approach has not yet been developed for other minerals and trace elements, and thus reported values for bioavailability from different studies are often not comparable.

Evidence from studies on experimental animals suggests that essentially all of the calcium in human milk, cow milk, and cow milk based infant formulas is potentially available for absorption in the gastrointestinal tract. Weeks and King (1985) found that net calcium absorption from cow's milk in 8- to 10-week-old rats, adapted to a low-calcium diet, was 100% in both ultra high temperature (UHT)- and high temperature–short time (HTST)-treated whole cow milk and that this high efficiency of absorption was maintained (~96%) after feeding for a further 6 weeks on the low-calcium diet. In 8- to 10-week-old rats receiving an adequate calcium diet, net absorption of calcium in both UHT and HTST milk was 85–88% and 6 weeks later was reduced to ~50%. This diminution was attributed to diminished need for calcium in calcium-adequate animals as skeletal mineralization was attained. These results show that calcium absorption from milk is determined by the need of the animal for calcium, and, when that need is high, essentially all of the calcium in cow milk is available for absorption (Morrissey and Flynn, 1991). These findings are supported by observations in the suckling rat in which mechanisms of homeostatic control of calcium absorption have not yet developed. Kunz and Lonnerdal (1990) showed that in 14-day-old suckling rats calcium absorption was 89% of dose for both human and cow milk and 92–94% from cow milk based infant formulas.

The work of Fomon *et al.* (1963) is often cited to suggest that the bioavailability of calcium in human milk is higher than in cow milk or cow milk based infant formulas. Fomon *et al.* (1963) reported that calcium was absorbed more efficiently by human infants from human milk than from cow milk or a cow milk formula with 738 mg Ca/liter. However, the absolute amount of calcium absorbed was greater from cow milk and formula, and calcium absorption from cow milk formulas with lower calcium contents (363–458 mg/liter) was similar to that from human milk.

Rudloff and Lonnerdal (1990a) found that calcium absorption in suckling rhesus monkeys (1.5–4 months old) was slightly lower for cow milk based infant formulas (66–74% of dose) than for human milk (84%). However, although the calcium content of these formulas was not reported, it seems likely that the absolute absorption of calcium from formulas was higher than from human milk, given the higher reported concentrations of calcium in infant formula (530–800 mg/liter) compared to human milk (Hurrell *et al.*, 1989a). These findings may explain the observation that bone mineralization is higher in infants fed on cow milk based infant formula than on human milk (Steichen and Tsang, 1987). Rudloff and Lonnerdal (1990b) reported that the efficiency of calcium absorption from human milk in weanling rhesus monkeys (7–8 months old) was significantly greater (72%) than from cow milk based infant formulas (45–53%), but absolute absorption from human milk and formulas was similar due to the higher calcium concentration in the formulas.

Mean calcium absorption from cow milk in healthy human adults has been variably reported as 21.4% (Griessen *et al.*, 1989), 27.6% (Heaney *et al.*, 1988), 25.7% (Tremaine *et al.*, 1986), 32.1% (Heaney and Weaver, 1990), and 45.5% (Fairweather-Tait *et al.*, 1989). Martin *et al.* (1990) reported that calcium absorption was similar from milk and $CaCO_3$ in pregnant women (36–47%), but was only 5–21% in postmenopausal women. However, Recker *et al.* (1988) reported that mean calcium absorption from cow milk was 26.7% (range 13–41%) of dose in healthy postmenopausal women. Tremaine *et al.* (1986) reported that calcium absorption from cow milk was higher (36.2% of dose) in β-galactosidase-deficient subjects than in a β-galactosidase-sufficient group (25.7%), and suggested that this reflected lower habitual calcium intake due to reduced milk consumption in the β-galactosidase-deficient subjects.

Thus, while it appears that all of the calcium in human milk, cow milk, and cow milk based infant formulas is potentially available for absorption, the absolute amount of calcium absorbed in animals or humans is determined by physiological factors, such as the efficiency of calcium absorption mechanisms in the gastrointestinal tract, which may be influenced by calcium needs, vitamin D status, and age, as well as by calcium concentrations in milk. In addition, some components of milk (lactose and phosphopeptides) may enhance calcium absorption.

Miller (1989) has recently reviewed the effect of lactose on calcium absorption. There is strong evidence that lactose promotes intestinal absorption (particularly in the ileum) and body retention of calcium in rats. This effect of lactose is independent of vitamin D, but the mechanism by which it occurs remains unresolved. Armbrecht and Wasserman (1976) suggested that undigested lactose reaching the ileum interacts with the brush border membrane, increasing its permeability to calcium. Lactose also appears to increase calcium absorption in human infants. Ziegler and Fomon (1983) showed that calcium absorption was significantly higher from a soy-based infant formula containing lactose (48%) than from a similar formula in which the carbohydrate source was a mixture of starch hydrolysate and sucrose (33%). However, studies on the effect of lactose on calcium absorption in human adults are conflicting. Tremaine *et al.* (1986) found no significant difference between calcium absorption from milk or lactose-hydrolyzed milk in either β-galactosidase-deficient or β-galactosidase-sufficient subjects, while Griessen *et al.* (1989) reported that calcium absorption was similar from milk (21.4%) and lactose-free milk (lactose replaced by glucose) (26.8%) in healthy adult subjects, but lactose increased calcium absorption in β-galactosidase-deficient subjects. Miller (1989) concluded that it is likely that lactose, at the levels normally present in milk, does not have a significant effect on calcium absorption by healthy adults consuming normal diets. It is possible that lactose affects the nonsaturable paracellular pathway for calcium absorption in the gut and its effect is most likely to occur in vitamin D deficiency, or when elevated levels of calcium are fed (Bronner, 1987; Miller, 1989).

It has been suggested that phosphopeptides formed during the digestion of bovine caseins may be involved in promoting calcium absorption (Mellander, 1950). Such phosphopeptides have the capacity to chelate calcium and to prevent the precipitation of calcium phosphate salts and may help to maintain a high concentration of soluble calcium in the intestinal lumen. There is evidence that phosphopeptides are present in the lumen of the small intestine of rats (Naito *et al.*, 1972) and minipigs (Meisel and Frister, 1989) after a casein meal, and purified phosphopeptides have been shown to enhance the absorption of calcium in rats (Sato *et al.*, 1986) and chicks (Mykkanen and Wasserman, 1980). However, the nutritional significance of these phosphopeptides in humans consuming milk remains unclear (West, 1991).

Age-related osteoporosis, a common bone disease and a major cause of disability in Western countries, is characterized by reduced bone density resulting in increased bone fragility and susceptibility to fracture. The condition is particularly common in elderly women, particularly Caucasians. It has been estimated that osteoporosis afflicts 24 million Americans (half of the women over 45 years of age and 90% of the women over 75 years of age) (NRC, 1989b).

Osteoporosis is a multifactorial disorder, but there is increasing evidence that inadequate calcium intake, particularly throughout early life, is a contributory

factor (Heaney *et al.*, 1982; Berner *et al.*, 1990). Adequate calcium intake is required in early life in order to develop maximum bone mass at maturity (age 30–40), and there is evidence that the amount of bone mass present at maturity is an important factor influencing fracture susceptibility in the elderly (Heaney *et al.*, 1982). Peak bone mass at maturity appears to be related to intake of calcium during the years of bone mineralization (NRC, 1989a,b; Berner *et al.*, 1990). The National Research Council (NRC, 1989a) has stated that the most promising nutritional approach to reduce the risk of osteoporosis in later life is to ensure a calcium intake that allows the development of each individual's genetically programmed peak bone mass during the formative years, i.e., throughout childhood to age 25 years. In this regard, the prevalence of lower than recommended calcium intakes among adolescent and young adult females in many countries, e.g., in the United States (Heaney *et al.*, 1982) and Europe (INDI, 1990), is of particular concern.

However, there is no clear evidence that bone density of postmenopausal women is related to concurrent dietary calcium intake within a wide range, except, perhaps, where this reflects a high-calcium intake pattern established in childhood (NRC, 1989a). Similarly, evidence that supplementary calcium can retard the rate of age-related bone loss after maturity is mixed (NRC, 1989a), and the effect of calcium, if any, is small (Kanis and Passmore, 1989). However, Nordin and Heaney (1990) have argued that calcium supplementation is beneficial and is justified by the available evidence.

Recently, attention has been focused on the possible effect of dietary calcium intake on blood pressure. Evidence from epidemiological studies and clinical intervention trials has led to the suggestion that (1) an abnormally low dietary intake of calcium (and by implication low intake of milk and dairy products) may contribute to the development of hypertension and (2) dietary supplementation with calcium will help to reduce established high blood pressure. This evidence has been critically reviewed by the British Nutrition Foundation's Task Force on Calcium (1989). It was concluded that the evidence for a role for calcium in the etiology of hypertension is weak and inconclusive and that the more recent carefully controlled intervention studies failed to confirm unequivocally earlier experiments that suggested that calcium supplementation can ameliorate hypertension.

V. MAGNESIUM

Magnesium has an essential role in a wide variety of physiological processes, including protein and nucleic acid metabolism, neuromuscular transmission, and muscle contraction, and it acts as a cofactor for many enzymes. Dietary deficiency of magnesium is uncommon except in conditions of severe malnutrition and

certain disease states (Shils, 1988). The RDAs for magnesium are 40 and 60 mg for infants 0–6 and 6–12 months old, respectively; 80, 120, and 170 mg for children aged 1–3, 4–6, and 7–10 years old, respectively; 270 and 280 mg for males and females aged 11–14 years old; 400 and 300 mg for males and females aged 15–18 years old; 350 and 280 mg for male and female adults; 300 mg for female adolescents and adults; and 300 mg in pregnancy and 355 mg in lactation (NRC, 1989a).

Mature human milk contains about 35 mg of magnesium per liter (AAP, 1985). Magnesium concentration has been reported to be about 30% higher in colostrum than in mature milk (Rajalakshimi and Srikantia, 1980), but it appears to be unaffected by stage of lactation in mature milk (Butte *et al.*, 1987; Vaughan *et al.*, 1979). There is no relationship between milk magnesium concentration and dietary magnesium intake within the normal range of dietary intake (Vaughan *et al.*, 1979; Kirskey *et al.*, 1979).

The mean concentration of magnesium in cow milk is 120 mg/liter, with a range of 90–140 mg/liter (Paul and Southgate, 1978). Magnesium concentrations in colostrum are two to three times those in mature milk and decrease to the level in mature milk within the first 1–3 days of lactation (Kirchgessner *et al.*, 1967), remaining relatively constant thereafter. Magnesium concentration in cow milk is unaffected by dietary magnesium intake (Kirchgessner *et al.*, 1967).

The distribution of magnesium in human milk has been reported as 2% in the fat fraction, 6% in casein, 36% associated with the whey proteins, and 58% in a low-molecular-weight form that has not been characterized (Fransson and Lonnerdal, 1983). In cow milk, all of the magnesium is in the skim milk phase (Fransson and Lonnerdal, 1983), where 65% of the magnesium is in a soluble form (40% as magnesium citrate, 7% as magnesium phosphate, and 16% as free magnesium ion), while the remainder is colloidal and is associated with the casein micelles (about half associated with colloidal calcium phosphate and half bound directly to phosphoserine residues in caseins) (Holt, 1985).

There is little information on the bioavailability of magnesium from milks in humans. Metabolic balance studies in infants showed that 16–43% of magnesium is absorbed from cow milk based infant formulas (Ziegler and Fomon, 1974) and that lactose enhances the absorption of magnesium (Ziegler and Fomon, 1983).

VI. PHOSPHORUS

Phosphorus is an essential nutrient for humans and serves a number of important biological functions. Phosphorus occurs as organic and inorganic phosphates in all body tissues and fluids, is an essential component of many biological

molecules, including lipids, proteins, carbohydrates, and nucleic acids, and plays a central role in metabolism. As calcium phosphate, it is a major structural component of bones and teeth. Because almost all foods contain phosphorus, dietary phosphorus deficiency does not usually occur (NRC, 1989a).

The RDAs for phosphorus are 300 and 500 mg for infants aged 0–6 and 6–12 months, respectively; 800 mg for children aged 1–10 years and adults over 25 years of age; and 1200 mg for adolescents and pregnant and lactating women (NRC, 1989a).

Cow milk and cow milk products, such as cheese and yogurt, are good dietary sources of phosphorus (Paul and Southgate, 1978), and the contribution of milk and dairy products to total phosphorus intake has been reported as 30–45% in Western countries (Renner, 1983; Hazell, 1985).

The mean phosphorus content in mature human milk (140 mg/liter) (AAP, 1985) is considerably lower than in cow milk (950 mg/liter; range, 900–1000 mg/liter) (Paul and Southgate, 1978). In human milk, phosphorus concentration increases from 100 mg/liter on day 1 to 170 mg/liter on day 8 and decreases to 130 mg/liter on day 36 of lactation (Harzer *et al.*, 1986a). There is little variation in the phosphorus content of cow milk throughout lactation (Holt, 1985).

In cow milk, 20% of the phosphorus occurs as organic phosphate esterified to casein, with the remainder as inorganic phosphate (Jenness, 1974). About 44% of inorganic phosphate is associated with casein micelles as calcium phosphate and 56% is soluble, mainly as free phosphate ions (Holt, 1985). In human milk, 23% of the phosphorus is protein bound (Harzer *et al.*, 1986b), about 15% occurs in an inorganic form, and the remainder is associated with the lipids (Renner, 1983).

In the first few weeks of life, the infant's ability to regulate plasma calcium concentration has not developed fully and hypocalcemia may result in neonatal tetany, which used to occur more frequently in artificially fed than in breast-fed infants. Excessive phosphorus intake contributes to this condition, and feeding unmodified cow milk, which is high in phosphorus, increases serum phosphorus and lowers serum (ionized) calcium in the newborn infant (DHSS, 1980). For this reason it is recommended that the calcium:phosphorus ratio in artificial infant formulas should be greater than that in cow milk (~1.2:1) and more similar to that in human milk (~2.2:1) (DHSS, 1980). Greer (1989) has suggested that the absolute amount of phosphorus in the formula is more important than the calcium:phosphorus ratio in the etiology of infantile tetany, and has proposed that the upper limit for phosphorus in formulas for full-term infants should be 490 mg/liter. Reported values for phosphorus in cow milk based formulas currently sold in the United States (mean, 362 mg/liter; range, 342–389 mg/liter) are lower than this proposed limit (Hamill *et al.*, 1989).

In experimental animals, a high intake of phosphorus or a low calcium:phosphorus ratio in the diet can lead to bone loss (Allen, 1982). However,

it is generally agreed that broad variations in phosphorus intake or in the calcium:phosphorus ratio in the diet do not adversely affect bone in adult humans (Heaney *et al.*, 1982).

VII. IRON

Iron, as a component of heme in hemoglobin, myoglobin, cytochromes, and other proteins, plays an essential role in the transport, storage, and utilization of oxygen (Bothwell *et al.*, 1979). It is also a cofactor for a number of enzymes. Deficiency of iron resulting in anemia afflicts about 30% of the world's population and occurs in both Western and underdeveloped countries (Baynes and Bothwell, 1990; Hallberg, 1982).

RDAs for iron are 6 mg for infants under 6 months of age; 10 mg for age 6 months to 10 years; 12 mg for male adolescents; 10 mg for male adults and postmenopausal females; 15 mg for females during adolescence, premenopausal adulthood, and lactation; and 30 mg in pregnancy (NRC, 1989a). Milk and milk products are very poor sources of iron, and cow milk contributes little to total iron intake (Pennington *et al.*, 1987; Hazell, 1985).

Mean iron concentration in mature human milk (0.3 mg/liter; AAP, 1985) is lower than that in cow milk (0.5 mg/liter; range, 0.3–0.6 mg/liter; Paul and Southgate, 1978). The iron content of human milk decreases with advancing lactation: 0.5–1.0 mg/liter in colostrum, decreasing to 0.2–0.4 mg/liter mature milk (Vaughan *et al.*, 1979; Vuori *et al.*, 1980; Siimes *et al.*, 1979). No correlation has been observed between the dietary intake of iron and its concentration in human milk (Vaughan *et al.*, 1979; Vuori *et al.*, 1980; Lonnerdal, 1986), and dietary supplementation with up to 30 mg of iron per day does not affect its concentration in milk (Siimes *et al.*, 1984; Picciano and Guthrie, 1976). Milk iron concentration is not affected by maternal iron status (Picciano and Guthrie, 1976).

Although several investigations have reported lack of a developmental pattern for iron in cow milk, de Maria (1978) showed that iron concentration decreased by 35–50% during the first 3 days of lactation and remained relatively constant thereafter. The iron content of cow milk is resistant to changes in dietary iron intake (Murthy, 1974; Murthy *et al.*, 1972). Contact with metal containers can increase the iron concentration in cow milk (Jarrett, 1979).

In cow milk, 14% of the iron occurs in milk fat (Fransson and Lonnerdal, 1983), where it is associated with the fat globule membrane. About 24% of the iron is bound to casein (Fransson and Lonnerdal, 1983), probably to the phosphoserine residues of caseins (Hegenauer *et al.*, 1979a), while 29% is bound to whey proteins and 32% is associated with a low-molecular-weight fraction (Fransson and Lonnerdal, 1983). The distribution of iron added to cow milk depends on

the form of the iron supplement used. Ferrous sulfate donates less iron to casein micelles than other supplements tested (Hegenauer *et al.*, 1979a). Supplementation of unhomogenized milk with ferrous salts results in a milk with a high iron content in the fat, which can result in lipid peroxidation and development of "off flavor" (Hegenauer *et al.*, 1979b,c). Homogenization, by causing absorption of casein onto the fat globule membrane, increases the affinity of milk fat for added iron. Ferric nitrilotriacetate donates iron specifically to the casein fraction, and iron added in this form causes relatively little lipid peroxidation (Hagenauer *et al.*, 1979b,c). Iron added as ferric chloride to skim milk is 85% bound to caseins (Demott and Dincer, 1976).

In human milk, 33% (16–46%) of the iron is associated with the lipid fraction (Fransson and Lonnerdal, 1980), and there is evidence that it is bound to xanthine oxidase, an enzyme that is a component of the fat globule membrane (Fransson and Lonnerdal, 1984). A significant proportion (~32%; range, 18–56%) of the iron is associated with a low-molecular-weight (<15,000) fraction that has not yet been characterized (possibly citrate; Lonnerdal, 1984), and only about 9% is bound to casein (Fransson and Lonnerdal, 1983). About 26% is bound to whey proteins, probably mainly to lactoferrin (Fransson and Lonnerdal, 1983), which is present in mature human milk at a concentration of 1.6–2 g/liter (McLelland *et al.*, 1978; Lonnerdal *et al.*, 1976) and is only 3–5% saturated with iron (Lonnerdal, 1984). Previously it had been reported that lactoferrin is saturated with iron to the extent of 10–30% (Bullen *et al.*, 1972). Considering the very high affinity of lactoferrin for iron ($K_{assoc} \sim 10^{30}\ M^{-1}$; Aisen and Leibman, 1972), it is difficult to understand why its degree of saturation with iron in milk is so low.

Lactoferrin is a glycoprotein (molecular weight ~86,000) that can bind two ferric ions and two carbonate or bicarbonate ions per molecule of protein. Its concentration in human milk is ~15 g/liter in colostrum, decreasing to ~5 g/liter on day 5 and 1.6–2 g/liter in mature milk (McLelland *et al.*, 1978; Lonnerdal *et al.*, 1976). The concentration of lactoferrin in bovine colostrum is ~6 g/liter, decreasing to ~0.9 g/liter in mature milk (Reiter, 1978; Meyer and Senft, 1979).

It has been suggested that lactoferrin in human milk, which is largely unsaturated with iron, has a bacteriostatic function, i.e., the strong affinity of lactoferrin for iron would enable this protein to sequester iron from its surroundings and thus prevent the growth of bacteria that require iron (Bullen *et al.*, 1972). While there is evidence that this does occur *in vitro* (Bullen *et al.*, 1972; Arnold *et al.*, 1977), definitive evidence that it occurs *in vivo* is still lacking (Brock, 1989). It is well established that the incidence of gastrointestinal infections is much lower in breast-fed than in bottle-fed infants (Cunningham, 1979; Fallott *et al.*, 1980), but whether this is influenced by lactoferrin is unknown, since other constituents of breast milk, such as immunoglobulins and enzymes, are probably involved also (Hambreus, 1977).

There is conflicting evidence regarding the possible role of lactoferrin in iron absorption. The mucosal surfaces of the human gastrointestinal tract are coated with a thin layer of lactoferrin (Masson *et al.*, 1969), and it has been suggested that lactoferrin may act by binding to specific receptors and donating iron to the small intestine and thus play a role in iron absorption (Cox *et al.*, 1979). The existence of a lactoferrin receptor that facilitates the uptake of lactoferrin-bound iron has been demonstrated in the small intestinal mucosae of the infant rhesus monkey (Davidsson and Lonnerdal, 1988), suggesting that this may represent a mechanism of lactoferrin–iron absorption. Although only a small portion of the iron in human milk is associated with lactoferrin (Fransson and Lonnerdal, 1980), it is possible that redistribution of iron from other ligands to lactoferrin may occur in the gastrointestinal tract (Lonnerdal, 1989a). Supplementation of weanling mice with lactoferrin-bound iron resulted in high iron uptake (Fransson *et al.*, 1983a), and Fransson *et al.* (1983b) found a significantly faster uptake of iron in pigs fed a formula with bovine lactoferrin–iron than in pigs fed a formula with ferrous sulfate. However, denaturation of lactoferrin in human milk by boiling does not alter the absorption of iron from human milk in adults, and human lactoferrin added to infant formula reduced iron absorption in human adults (McMillan *et al.*, 1977). Fairweather-Tait *et al.* (1988) have shown that bovine lactoferrin does not facilitate the uptake of lactoferrin–iron in human infants, although this could be due to the species specificity of mucosal receptors for lactoferrin (Lonnerdal, 1989a). Brock (1980) suggested that lactoferrin may suppress the absorption of iron in the young infant and prevent iron overload, and may thus regulate iron absorption. Further studies are needed to establish whether lactoferrin in human milk plays a role in iron absorption in infants.

Bioavailability of iron to the infant fed human milk is reported to be in the range 49–70% (Saarinen *et al.*, 1977; Saarinen and Siimes, 1979). Considerably lower absorption efficiency, usually about 10–34%, of iron from cow milk has been reported in human infants (Saarinen *et al.*, 1977; Saarinen and Siimes, 1979; McMillan *et al.*, 1976; Schulz and Smith, 1958). To compensate for the relatively low bioavailability of iron in cow milk, infant formulas are often supplemented with iron at levels of 5–8 mg/liter in Europe (Muzzarelli *et al.*, 1983) and 12 mg/liter in the United States (Dallman, 1989), although nonsupplemented formulas may be used provided they contain a minimum of 1 mg Fe/liter (AAP, 1976b) or 0.7 mg/liter (ESPGAN, 1977; DHSS, 1980). In infants, iron absorption from cow milk formula with 12 mg Fe/liter as ferrous sulfate is about 4–7% (Rios *et al.*, 1975; Saarinen and Siimes, 1977), but because of the much higher concentration of iron in such formulas, the absolute amount of iron absorbed is considerably greater than is absorbed from human milk.

The reason for the exceptionally high bioavailability of iron in human milk is not understood. It has been suggested that it may be related to the high concentration of lactoferrin in human milk, but, as previously discussed, evidence

for a role for lactoferrin in iron absorption is conflicting. Others have suggested that the apparent higher bioavailability of iron in human milk compared to cow milk could be due to a number of factors, including a lower concentration of proteins, calcium, and phosphorus (potential inhibitors of absorption) and higher concentrations of lactose and ascorbate (enhancers of iron absorption) in human milk (McMillan *et al.*, 1976; Lonnerdal, 1985; Hurrell *et al.*, 1989b). However, studies on suckling rats, which have a very high capacity for iron absorption, have shown that a very high and similar proportion (~90%) of Fe is absorbed from human milk, cow milk, and iron-supplemented cow milk based formula (Brennan *et al.*, 1989, 1990a,b; Flynn and Brennan, 1991), suggesting that there are no unabsorbable iron complexes in these milks. It is likely that the lower concentration of iron in human milk may be a contributory factor to the higher absorption efficiency of iron from human milk in infants, since iron absorption is subject to homeostatic control and small amounts are absorbed more efficiently than large amounts.

There has been considerable debate on whether the iron content of human milk is sufficient for the breast-fed infant and whether these infants should be supplemented with iron. Iron deficiency is one of the most common nutritional deficiencies in infancy and childhood due to rapid growth and marginal supplies of iron in the diet (Dallman *et al.*, 1980). It has been suggested that the iron stores of a breast-fed infant 4–6 months of age may become compromised if they are not replenished from dietary sources, and dietary iron supplementation has been recommended at no later than 4 months of age for full-term infants and no later than 2 months of age for premature infants (AAP, 1976a). However, the iron status of infants after 6 months of exclusive breast feeding has been reported to be similar to that of infants receiving iron-supplemented infant formulas (McMillan *et al.*, 1976; Saarinen, 1978; Woodruff *et al.*, 1977), and there is evidence that the amount of iron in human milk and the considerable stores of iron present at birth are sufficient to meet the needs of full-term infants for the first 6 months of life (Duncan *et al.*, 1985; Siimes *et al.*, 1984; NRC, 1989a; Saarinen, 1978).

Iron-fortified cow milk based formulas are effective in preventing iron deficiency (Dallman, 1989), which may be partly attributable to the fact that ascorbate is also added to formulas at levels that markedly enhance the absorption of fortification iron (Stekel *et al.*, 1986). Dallman (1989) has attributed the marked decline in the prevalence of anemia in infants and preschool children in the United States over the past two decades to the increased use of iron- and ascorbate-fortified infant formulas.

Concern has been expressed regarding present levels of iron fortification of infant formulas in the United States (Dallman, 1989), in particular in relation to the possibility that such high intakes of iron might impair nutritional immunity

and predispose to infection. However, no evidence of an effect of iron fortification on prevalence of infection has been demonstrated (Dallman, 1989).

Formula without added iron is preferable for the first 2–4 weeks in very low-birth-weight infants due to the susceptibility of these infants to hemolytic anemia resulting from iron-catalyzed damage to the red blood cell membrane. It appears that the occurrence of this condition in the past was due to a combination of factors in addition to high iron intake, i.e., vitamin E deficiency in the infants and high levels of polyunsaturated fatty acids in the formulas (Anonymous, 1988a).

There is lack of agreement on when it is appropriate to introduce cow milk into the diet of the infant, particularly in relation to its possible effect on iron status. The American Academy of Pediatrics (AAP, 1983) has recommended that cow milk may be introduced after 6 months of age. However, there is evidence that infants fed cow milk at 6 months have, by 12 months of age, diminished reserves of iron and a higher incidence of iron deficiency than do formula-fed infants (Ziegler, 1989). Reservations regarding the introduction of cow milk during the first year have been expressed (Oski, 1989; Ziegler, 1989), since it is a poor source of iron and replaces iron-rich foods in the infant's diet, increases intestinal blood loss (when compared to heat-processed cow milk based formula), and may inhibit iron absorption. The relative importance of these factors in contributing to the poor iron nutritional status in cow milk fed infants is unkown (Ziegler, 1989). The mechanism by which whole cow milk increases gastrointestinal blood loss is unclear. It has been suggested that it may be due to a milk protein component that can be modified by heat processing (Fomon *et al.*, 1981).

VIII. ZINC

Zinc is essential for growth and development, sexual maturation, and wound healing, and it may also be involved in the normal functioning of the immune system and other physiological processes. It is a component of the hormone insulin and aids in the action of a number of hormones involved in reproduction, as well as being required for the synthesis of DNA, RNA, and protein and as a cofactor for many enzymes involved in most major metabolic processes (Hambidge *et al.*, 1986). Zinc deficiency in humans was first reported in the Middle East in the early 1960s (Prasad, 1982), giving rise to dwarfism, impaired sexual development, and anemia. Mild deficiencies of zinc, although difficult to detect, have been shown to occur in Western countries, particularly in infants and young children (Hambidge *et al.*, 1972; Walravens *et al.*, 1983, 1989), giving rise to low hair zinc levels, suboptimal growth, poor appetite, and impaired taste acuity.

RDAs for zinc are 5 mg for infants aged 0–12 months, 10 mg for children aged 1–10 years, 15 mg for male adolescents and adults, 12 mg for female adolescents and adults, 15 mg in pregnancy, and 19 mg during the first 6 months of lactation (NRC, 1989a).

Dairy products such as milk, cheese, and yogurt are moderately good sources of zinc, and it has been estimated that milk and dairy products contribute between 19 and 31% of the total zinc intake in Western countries (Hazell, 1985; Renner *et al.*, 1989).

Mean zinc concentration in mature human milk during the first 6 months of lactation (1.2mg/liter; AAP, 1985) is considerably lower than that in cow milk (3.5–3.8 mg/liter; Paul and Southgate, 1978; Pennington *et al.*, 1987), but large variations in the zinc content of human (0.65–5.3 mg/liter; Lonnerdal *et al.*, 1981) and cow milk (2.0–6.0 mg/liter; Paul and Southgate, 1978) have been reported. There is a pronounced decrease in the zinc concentration in human milk during lactation (Casey *et al.*, 1985, 1989; Vuori and Kuitunen, 1979). For example, Casey *et al.* (1985, 1989) reported that zinc concentration decreases from 11.5 mg/liter at 2 days to 4.6 mg/liter at 7 days, 3.0 mg/liter at 1 month, and 0.5 mg/liter at 12 months lactation. No significant correlation was observed between dietary zinc intake and zinc concentration in human milk (Moser and Reynolds, 1983; Vuori *et al.*, 1980), and zinc supplementation of a zinc-adequate diet does not appreciably affect the zinc concentration in milk (Karra *et al.*, 1989; Kirskey *et al.*, 1979; Picciano and Guthrie, 1976). There is a large decrease (50%) in the zinc concentration in cow colostrum during the first 3 days of lactation, with little change thereafter (de Maria, 1978). Dietary zinc supplementation increases the zinc concentration of cow's milk only slightly (Murthy, 1974).

There are significant differences in the distribution of zinc in human and cow milks. In cow milk, most of the zinc is in the skim milk fraction, with only 1% in the lipid fraction (Fransson and Lonnerdal, 1983). Of the zinc in the skim milk fraction, over 95% is associated with the casein micelles (Blakeborough *et al.*, 1983; Singh *et al.*, 1989b), with small proportion (~5%) associated with a low-molecular-weight compound (Singh *et al.*, 1989b) that has been identified as citrate (Martin *et al.*, 1984; Blakeborough *et al.*, 1983). Within the casein micelles, one-third of the zinc is loosely bound to casein phosphoserine residues and two-thirds is more tightly bound to colloidal calcium phosphate (Singh *et al.*, 1989a,b).

Lonnerdal *et al.* (1982) reported that zinc in human milk is distributed as follows: 29% (20–45%) in the lipid fraction, bound to the fat globule membrane (Frannson and Lonnerdal, 1983); 14% (5–21%) associated with casein; 28% (7–48%) with whey proteins (serum albumin; Lonnerdal *et al.*, 1982); and 29% (24–36%) with a low-molecular-weight compound, probably citrate. The association of a significant proportion of zinc in human milk with a low-molecular-

weight compound has been observed in a number of studies (Hurley *et al.*, 1977, 1979; Eckhert *et al.*, 1977; Cousins and Smith, 1980). The identity of this low-molecular-weight zinc-binding ligand in human milk has been the subject of much controversy. Evans and Johnson (1980) suggested that picolinic acid is the ligand, but this has been discounted by Rebello *et al.* (1982), who found that the picolinic acid concentration in human milk is insufficient to bind a significant proportion of the zinc, and by Hurley and Lonnerdal (1981), who found that picolinic acid does not bind zinc in human milk. The ligand has been identified by a number of workers as citrate (Lonnerdal *et al.*, 1980; Martin *et al.*, 1981, 1984).

A number of lines of evidence suggest that the bioavailability of zinc in human milk is greater than in cow milk. Human milk (but not cow milk) has a therapeutic value in the treatment of acrodermatitis enteropathica, a hereditary zinc malabsorption syndrome (Moynahan, 1974; Hurley *et al.*, 1979; Eckhert *et al.*, 1977). The plasma zinc concentration of breast-fed infants has been reported to be significantly higher than that in infants fed a cow milk based formula containing 1.8 mg zinc/liter (Hambidge *et al.*, 1979). Studies in human adults using extrinsic labeling with ^{65}Zn, with whole body counting (Sandstrom *et al.*, 1983a; Lonnerdal *et al.*, 1984), showed that zinc absorption from human milk (41 ± 9%) was significantly greater than from cow milk (28 ± 15%) or cow milk based infant formula (31 ± 7%). Short-term studies on human adults (Casey *et al.*, 1981), pigs (Blakeborough *et al.*, 1986), and suckling rats (Sandstrom *et al.*, 1983b) show that zinc is absorbed more rapidly from human milk than from cow milk or cow milk based infant formulas.

A number of studies have failed to show a higher bioavailability of zinc from human milk than from cow milk or formulas. Johnson and Evans (1978) reported that the bioavailability to rats of zinc from human and cow milk was 59 and 42%, respectively, but this difference was not statistically significant. Lonnerdal *et al.* (1988) reported that zinc absorption in suckling rhesus monkeys was not significantly higher from human milk (65% of dose) or from whey-predominant formula (60%) than from casein-predominant formula (46% of dose). In fact, the absolute absorption of zinc was considerably higher from the formulas than from human milk due to the higher zinc concentrations in these formulas.

There are two main theories that have been proposed to explain the higher bioavailability of zinc from human milk compared with cow milk: (1) Binding of a significant fraction of the zinc in human milk (but not in cow milk) to a low-molecular-weight zinc-binding ligand (e.g., citrate) may enchance zinc absorption either by facilitating zinc transport across the gastrointestinal tract wall or by preventing the sequestration of zinc by other substances in the gastrointestinal tract, which would lower its availability for absorption (Hurley *et al.*, 1977; Eckhert *et al.*, 1977). (2) Binding of a large fraction of zinc in cow milk to casein [present at about 10 times its concentration in human milk (Hambreus,

1977)] may result in the entrapment of zinc in casein curds formed in the stomach, which may be incompletely digested in the small intestine, thus rendering a significant proportion of the zinc unavailable for absorption (Blakeborough *et al.*, 1983, 1986; Harzer and Kauer, 1982; Lonnerdal *et al.*, 1980). However, studies of suckling rats, which have a high capacity for zinc absorption, showed that a very high and similar proportion of zinc (85–95%) was absorbed from human milk, cow milk, and cow milk based infant formulas, suggesting that there are no unabsorbable forms of zinc in either human or cow milk (Brennan *et al.*, 1989, 1990a,b; Flynn and Brennan, 1991). It is likely that the lower concentration of zinc in human milk may be a contributory factor to the higher absorption efficiency of zinc from human milk in infants, since zinc absorption is subject to homeostatic control and small amounts are absorbed more efficiently than large amounts.

Walravens and Hambidge (1976) showed that supplementation of infant formulas to increase zinc levels from 1.8 to 5.8 mg/liter resulted in increased growth rates in male, but not in female, infants. These findings suggested that the zinc content of unsupplemented cow milk formula is insufficient for infants, and zinc supplementation of formulas to a minimum of 3.2 mg/liter is now recommended (AAP, 1976b). Supplementation is now widely practiced, as is evident from a recent report showing that the mean zinc content of cow milk based infant formulas in the United States is 6 mg/liter (range, 4.0–7.4 mg/liter) (Hamill *et al.*, 1989).

IX. COPPER

Copper is an essential element for a wide range of animal species (Davis and Mertz, 1987). It is required for iron utilization and is a cofactor for enzymes involved in the metabolism of glucose and the synthesis of hemoglobin, connective tissue, and phospholipids. Dietary deficiency of copper is uncommon except in conditions of severe malnutrition (Danks, 1988). Dietary requirements for copper are not known precisely, but estimated safe and adequate daily intakes are 0.4–0.6 and 0.6–0.7 mg for infants aged 0–6 months and 6–12 months, respectively; 0.7–1.0, 1.0–1.5, and 1.0–2.0 mg for children aged 1–3, 4–6, and 7–10 years, respectively; and 1.5–3.0 mg for adolescents and adults (NRC, 1989a).

Milk and milk products are considered poor sources of copper, and cow milk contributes little to the total dietary intake of copper (Pennington *et al.*, 1987; Renner *et al.*, 1989; Spring *et al.*, 1979). The mean copper concentration in mature human milk (0.25 mg/liter; AAP, 1985) is higher than in cow milk (0.09 mg/liter; Pennington *et al.*, 1987; Varo *et al.*, 1980). In human milk, copper

concentration decreases during lactation; mean values decrease from 0.6mg/liter in the first and second week of lactation (Vuori and Kuitunen, 1979; Casey *et al.*, 1989) to 0.36 mg/liter at 6–8 weeks (Vuori *et al.*, 1980) and to 0.21–0.25 mg/liter at about 20 weeks (Casey *et al.*, 1989; Vuori and Kuitunen, 1979; Vuori *et al.*, 1980). There is no significant correlation between dietary copper intake and milk copper concentrations (Kirskey *et al.*, 1979; Vaughan *et al.*, 1979; Vuori *et al.*, 1980).

Copper concentration in cow milk decreases by up to 50% during the first 3 days of lactation (de Maria, 1978), but can be increased by dietary copper supplementation (Murthy, 1974) or by contact with metal containers and processing equipment (Roh *et al.*, 1976). Contamination of cow milk with copper from brass containers has been cited as the major environmental factor in the etiology of Indian childhood cirrhosis (Tanner *et al.*, 1983; Bhave *et al.*, 1987).

The distribution of copper in human milk has been reported by Lonnerdal (1985) and Lonnerdal *et al.* (1982) to be 9–15% in the fat fraction, 39–56% bound to whey proteins (mainly albumin), 7–28% bound to casein, and 21–24% in a low-molecular-weight form. In cow milk, copper distribution has been reported as 2% in the fat fraction, 8% bound to whey proteins, 44% to casein, and 47% in a low-molecular-weight fraction (Lonnerdal, 1985). Martin *et al.* (1984) have shown that a significant amount of copper in human milk is associated with low-molecular-weight ligands (mainly citrate, but also including glutamate and other amino acids).

The average daily intake of copper by exclusively breast-fed North American infants is 0.23 mg over the first 4 months of lactation (Butte *et al.*, 1987). Copper deficiency is rare in breast-fed infants (Lonnerdal *et al.*, 1982), but copper deficiency has been reported in formula-fed infants in some countries (Tanaka *et al.*, 1980). Salmenpera *et al.* (1989) observed that plasma copper and ceruloplasmin in healthy full-term infants fed on cow milk formula (containing 0.08 mg copper/liter) are resistant to dietary supplementation with copper and are similar to values in breast-fed infants, suggesting that these infants have adequate copper status. Feeding infant formula with 0.03 or 0.4 mg/liter copper has been shown to yield normal parameters of copper status, similar to those of breast-fed infants (Salim *et al.*, 1986). The recommended minimum copper content of infant formulas is 0.4 mg/liter in the United States (AAP, 1976b) and 0.2 mg/liter in Europe (ESPGAN, 1977). Infant formulas are normally supplemented with copper to 0.4–0.6 mg/liter. The mean copper content of cow milk based infant formulas sold in the United States was reported as 0.69 mg/liter (Hamill *et al.*, 1989).

Knowledge of copper absorption from milks is very limited. In a study in suckling rats, copper absorption was 83% from human milk, 76% from cow milk, and 86–87% from cow milk based formulas, suggesting that there are no unabsorbable forms of copper in these milks (Lonnerdal *et al.*, 1985a).

X. MANGANESE

Manganese is an essential element for every animal species studied (Hurley and Keen 1987). It is a specific cofactor for glycosyl transferases, which are involved in mucopolysaccharide synthesis, and a nonspecific cofactor for a wide variety of other enzymes (Anonymous, 1988b). There are two known manganese metalloenzymes: pyruvate carboxylase and superoxide dismutase (Hurley and Keen, 1987). Manganese is widely distributed in foods, and dietary deficiency is not known to occur in humans.

The exact dietary requirements for manganese are unknown, but recommended safe and adequate daily intakes are 0.3–0.6 and 0.6–1.0 mg for infants aged 0–6 and 6–12 months, respectively; 1.0–1.5, 1.5–2.0, and 2.0–3.0 mg for children aged 1–3, 4–6, and 7–10 years, respectively; and 2.5–5.0 mg for adolescents and adults (NRC, 1989a).

Cow milk is a poor source of manganese and contributes little (1–3% in Western countries) to the total dietary intake of this mineral (Hazell, 1985; Jarrett, 1979). The mean manganese concentration in cow milk is 30 μg/liter (Lonnerdal *et al.*, 1981). The manganese concentration is higher in colostrum (100–160 μg/liter) than in mature milk (20–50 μg/liter) (de Maria, 1978; Archibald, 1958), and a decrease of over 50% has been reported to occur during the first 3 days of lactation (de Maria, 1978). Oral supplements of manganese given to cows can increase the manganese content of milk, provided that large doses are administered over a long period of time (Archibald, 1958).

The mean concentration of manganese in mature human milk is 6 μg/liter (AAP, 1985). Manganese concentration decreases with stage of lactation (Casey *et al.*, 1989; Stasny *et al.*, 1984; Vuori, 1979). Stasny *et al.* (1984) reported that manganese content decreased from 6.6 μg/liter during the first month of lactation to 3.5 μg/liter by the third month. A correlation between dietary manganese intake and milk manganese has been reported (Vaughan *et al.*, 1979; Vuori *et al.*, 1980).

The distribution of manganese in human milk has been reported as 67% bound to lactoferrin, 11% to caseins, 18% to the fat globule membrane, and 4% in a low-molecular-weight form that was not identified (Lonnerdal *et al.*, 1985b). In cow milk, manganese distribution was 67% bound to caseins, 1% to the fat globule membrane, 14% to whey proteins, and 18% to a low-molecular-weight fraction (Lonnerdal *et al.*, 1985b).

Daily manganese intake of fully breast-fed infants between 1 and 3 months of age is about 2.3–3.6 μg (Vuori, 1979; Stasny *et al.*, 1984), and this appears to be adequate since no cases of manganese deficiency in human infants have been reported (Lonnerdal *et al.*, 1983). A wide variation in the manganese content of infant formulas has been reported, with concentrations in several formulas 100–1000 times higher than in human milk (Lonnerdal *et al.*, 1983; Stasny *et*

al., 1984). These authors suggested that long-term intake of such formulas could lead to manganese toxicity, although no such effects have been reported. The higher levels were as a result of supplementation that has now ceased in the United States (Lonnerdal, 1989b). Even without manganese supplementation, infant formulas contain 50–300 μg/liter (Lonnerdal, 1989b). Hamill *et al.* (1989) reported that the range of manganese contents of milk based infant formulas in the United States decreased from 46–1427 μg/liter in 1981–1983 to 45–261 μg/liter in 1984–1985. Data from Muzzarelli *et al.* (1983) indicate that the manganese content of infant formulas in European countries ranges from 23 to 208 μg/liter. The American Academy of Pediatrics (AAP, 1976b) has recommended that the minimum manganese content of infant formulas for healthy full-term infants be 34 μg/liter and it has been proposed that the upper limit of manganese in infant formulas should be 335–600 μg/liter (Hambidge and Krebs, 1989; Lonnerdal, 1989b).

Very little is known about manganese bioavailability in milks. Davidsson *et al.* (1989) reported that fractional manganese absorption in healthy adults from human milk (8.2 ± 2.9%) was significantly higher than from cow milk (2.4 ± 1.7%), while absorption from cow milk based infant formulas was 1.7–5.9%. However, the absolute amount of manganese absorbed from cow milk and formulas was greater than from human milk due to the higher manganese concentrations in these milks. Studies with suckling rats showed that manganese absorption was not significantly different from human milk (81%) compared to cow milk (89%) or cow milk based formulas (80%) (Keen *et al.*, 1986), suggesting that there are no unabsorbable forms of manganese in these milks.

XI. SELENIUM

Selenium is an essential component of the enzyme glutathione peroxidase, which occurs in many human tissues where, together with vitamin E and the enzymes catalase and superoxide dismutase, if functions as an antioxidant, protecting cells against oxidative damage (Hoekstra, 1975). The RDAs for selenium are 15, 20, and 30 μg for children aged 0.5–1, 1–6, and 7–10 years, respectively; 40–50 μg for adolescents; and 55–70 μg for adults (NRC, 1989a).

In areas of China where the soil content of selenium is low, selenium deficiency causes Keshan disease, a cardiomyopathy that affects primarily young children and women of childbearing years (Chen *et al.*, 1980). Low selenium status has also been reported in New Zealand and Finland (Diplock, 1987), countries where the soil content of selenium is also low. There have been reports of low selenium status in patients receiving intravenous feeding solutions used in total parenteral

nutrition; these solutions contain very little selenium (Levander and Burk, 1986), and some cases of cardiomyopathy have occurred (Fleming *et al.*, 1982).

The contribution of dairy products to daily dietary intake of selenium has been estimated as 5 μg (8% of the total intake) in the United Kingdom (Thorn *et al.*, 1978), 13 μg (10%) in the United States (Morris and Levander, 1970), 13 μg (21–26%) in Finland (Varo and Koivistoinen, 1981), and 11 μg (29%) in New Zealand (Thomson and Robinson, 1980). The mean selenium concentration in whole cow milk in the United States studied as part of the Food and Drug Administration's Total Diet Study from 1975 through 1985, was 10 μg/liter (Pennington *et al.*, 1987). Mean selenium concentration in cow milk samples from 15 countries was reported as 10 μg/liter (range, 3–40 μg/liter) (Varo *et al.*, 1984). The concentration in milk depends on dietary intake, and in areas such as New Zealand and Finland, where the selenium content of soil and plants is low, concentrations as low as 3–5 μg/liter have been reported (Grant and Wilson, 1968; Millar and Sheppard, 1972; Millar *et al.*, 1973; Varo *et al.*, 1984; Varo and Koivistoinen, 1981). Maus *et al.* (1980) showed that the selenium content of cow milk increased linearly from about 30 to 55 μg/liter when dietary selenium was increased from about 2 to 6 mg/day.

The mean selenium content of mature human milk in the United States has been reported as 16 μg/liter (range, 8–34 μg/liter) (Smith *et al.*, 1982), whereas in the United Kingdom, mature human milk contains an average of 14 μg/liter of selenium, with a range of 8–19 μg/liter for five pooled samples (DHSS, 1977). Lower values have been reported in milk from women in countries where the soil content of selenium is low, such as New Zealand (7.6 μg/liter; Williams, 1983), Finland (6 μg/liter; Kumpulainen *et al.*, 1983), and Belgium (9.4 μg/liter; Robberecht *et al.*, 1985). Selenium concentration is higher in human colostrum (41 μg/liter) than in mature milk (16 μg/liter) (Smith *et al.*, 1982), and Levander *et al.* (1987) found that the selenium content of human milk fell from 20 μg/liter at 1 month postpartum to 15 μg/liter at 3 and 6 months postpartum. Milk selenium content is positively correlated with maternal plasma selenium concentration and plasma glutathione peroxidase (GSH-Px) activity (Mannan and Picciano, 1987; Levander *et al.*, 1987), indicating that milk selenium content is influenced by maternal selenium status. Kumpulainen *et al.* (1984) reported that the decline in milk selenium concentration with stage of lactation in Finnish women was much less marked in 1980 (11.8, 10.9, and 10.0 μg/liter at 1, 2, and 3 months postpartum, respectively), when average selenium intake was 50 μg/day, than in 1976 (10.7 and 5.8 μg/liter at 1 and 3 months, respectively), when average selenium intake was 33 μg/day. Kumpulainen *et al.* (1985) reported that supplementing lactating Finnish mothers with 100 μg selenium as selenite or yeast increased serum selenium and milk selenium concentrations.

Milner *et al.* (1987) reported that all the selenium in human milk was associated with protein and was precipitated by acetone (55%). Approximately 5% of the selenium in human milk was associated with the lipid fraction. The dialyzable

selenium ranged from 0 to 40% of total selenium, but the nature of the dialyzable fraction was not determined. At least nine selenoproteins ranging from 8 to >150 kDa, one of which was GSH-Px (~95 kDa), were detected in dialyzed human milk by molecular sieve chromatography. GSH-Px accounted for 15–30% of total selenium, similar to the value of 23% reported by Hojo (1982) and 20–25% found by Debski *et al.* (1989). Hojo (1982) showed that about 12% of milk selenium was accounted for by GSH-Px in cow milk.

The selenium requirement of the human infant is not known, but a dietary allowance of 10 μg has been recommended for infants under 6 months of age (NRC, 1989a). The average selenium content of milk of North American women is considered ample for breast-fed infants (NRC, 1989a). Zabel *et al.* (1978) reported that the selenium content of cow milk based infant formulas used in the United States was 5–12 μg/liter and a mean selenium concentration of 6.7 μg/liter (range, 5.1–9.2 μg/liter) has been reported by Smith *et al.* (1982), which is significantly lower than that in mature human milk. Mean daily selenium intake of formula-fed infants in the United States at 3 months of age (~7.2 μg) has been reported to be significantly lower than that of breast-fed infants (~10.1 μg), and selenium status, as reflected by serum selenium concentrations, was lower in the formula-fed infants (Smith *et al.*, 1982). Kumpulainen *et al.* (1987) showed that the selenium status of exclusively breast-fed infants from 3 to 9 months of age in Finland was higher than that of infants fed a cow's milk-based formula containing 3–5 μg selenium/liter, but lower than that of infants fed a the same formula containing 20 μg selenium/liter. However, there was no significant difference in selenium status of the three groups at 12 months of age. Litov *et al.* (1989) showed that in the United States, selenium status from birth to 2 months of term infants fed whey-predominant infant formula containing only intrinsic selenium (13 μg/liter), or the same formula with added sodium selenite to give a total selenium content of 34 μg/liter, was similar to that of infants fed on human milk (23 μg selenium/liter). Levander (1989) has proposed that infant formulas should contain selenium sufficient to provide 10–45 μg selenium/day. This suggests that infant formulas may need to be supplemented with selenium. Further research is needed on which chemical form of selenium is most appropriate for this purpose, particularly in relation to nutritional bioavailability. The bioavailability of selenium from human and cow milk is unknown, although Kumpulainen *et al.* (1985) has suggested that the bioavailability of breast milk selenium is high.

XII. IODINE

Iodine is an essential component of the thyroid hormones, thyroxine and triiodothyronine, which are important in controlling the rate of basal metabolism and in reproduction (Hetzel and Maberly, 1986). Dietary deficiency of iodine

causes enlargement of the thyroid gland and goiter, while a large excess of iodine in the diet reduces the uptake of iodine by this gland and also produces signs of thyroid deficiency (thyrotoxicosis). In 1983, there were an estimated 400 million iodine-deficient persons in the less developed regions of the world (Hetzel and Maberly, 1986) and an estimated 112 million in the more developed areas (Matovinovic, 1983). The occurrence of iodine toxicity has been reviewed recently (Pennington, 1990).

RDAs for iodine are 40 μg for infants under 6 months of age; 50 μg for infants aged 6–12 months; 70, 90, and 120 μg for children aged 1–3, 4–6, and 7–10 years, respectively; 150 μg for adolescents and adults; and 175 and 200 μg during pregnancy and lactation, respectively (NRC, 1989a). Daily intakes of up to 1000 μg are considered safe for adults (NRC, 1970).

The mean iodine content of mature human milk in the United States has been reported as 142 μg/liter (range, 21–281 μg/liter) (Bruhn and Franke, 1983) and 178 μg/liter (range, 29–400 μg/liter) (Gushurst *et al.*, 1984), while mean values of 70–90 μg/liter (range, 20–330 μg/liter) have been reported in Europe (DHSS, 1977; Delange *et al.*, 1988). There is a correlation between the iodine content of milk and dietary iodine intake (Gushurst *et al.*, 1984), and the use of iodized salt by the mother can increase milk iodine concentration (AAP, 1981). It has been estimated that an average daily breast milk intake of 700 ml would provide about 56 μg of iodine in Europe and 112 μg in the United States (Fisher, 1989). The amounts of iodine in the milk of North American women are much greater than the needs of their infants and reflect the elevated iodine intake of the mothers (Gushurst *et al.*, 1984).

Recommended levels of iodine in infant formulas in the United States are 34–500 μg/liter (AAP, 1985). It has been proposed recently that the upper limit of iodine in infant formulas should be 350 μg/liter (Fisher, 1989), reflecting concern that iodine-induced hypothyroidism might occur in infants consuming formulas with the higher level. Summarized data from various countries on the iodine content of cow milk based infant formulas indicate mean values of 107–376 μg/liter (Pennington, 1988). Representative values for the iodine content in cow milk based infant formulas sold in the United States are 30–150 μg/liter (Picciano, 1985).

Summarized data from various countries on the iodine content of cow milk indicate mean values of 100–770 μg/liter and a wide range of individual values, from 20 to >4000 μg/liter (Pennington, 1988). In the literature prior to 1970, iodine in milk was rarely reported to exceed 100 μg/liter (Hemken, 1980). Pennington *et al.* (1987) reported that the iodine content of whole cow milk analyzed as part of the FDA's Total Diet Study from 1975–1985 was 350 ± 237 μg/liter (range, 21–970 μg/liter). The values for milk collected in the early 1980s showed a tendency to be lower than those for milk collected in the late 1970s. The average iodine content of cow milk in the United States in

1980–1981 was 338 μg/liter in California and 514 μg/liter in milk collected in several other states (Bruhn and Franke, 1985), and a mean iodine content of 394 μg/liter was reported for milk in New York in the early 1980s (Dellavalle and Barbano, 1984). The mean iodine content of milk decreased to 178 μg/liter in California farm milk in 1985–1986 as a result of improved farm management practices (Bruhn *et al.*, 1987).

The iodine concentration in cow milk is influenced by season and is closely related to dietary intake, and feeding winter rations containing mineral supplements results in considerable increases in milk iodine (Broadhead *et al.*, 1965; Miller *et al.*, 1975). High concentrations of iodine in cow milk have been related to the addition of excessive amounts of ethylenediamine dihydriodide (EDDI) to dairy cow rations (Franke *et al.*, 1983; Hemken *et al.*, 1981). Dietary supplementation with inorganic iodine as potassium iodide does not increase the iodine content of milk as much as organic iodine fed as EDDI.

The use of iodophors for teat disinfection, if used as recommended, increases the iodine content in cow milk by 100–150 μg/liter (Hemken *et al.*, 1981), and by 300–700 μg/liter with improper use (Harding, 1982). For example, in Australia, milk from dairy farms where iodophors were not in use had a mean iodine concentration of 37 μg/liter, but milk from farms using iodophors had a mean iodine concentration of 760 μg/liter (Dunsmore, 1976). Iodine contamination of milk occurs as a result of absorption through the skin rather than by surface contamination of the teat (Conrad and Hemken, 1978). Use of iodophors for the disinfection of containers, milking machines, and processing equipment can also cause iodine contamination of milk (Harding, 1982). Harding (1982) has recommended the standardization of mineral feed supplements and supervised and restricted use of iodophor disinfectants as measures to reduce the iodine content of milk and milk products.

The increasing iodine content of cow milk in some countries during the 1960s and 1970s contributed significantly to increased iodine intakes, to levels sufficient to give rise to health concerns (Hemken, 1980; Park *et al.*, 1981). Nelson and and Phillips (1985) and Phillips *et al.* (1988) reported that seasonal variations in dietary iodine intake in Cambridge, England were mainly due to seasonal differences in iodine content of milk. Total milk and milk products provided 53–61% of iodine intake in winter and 24–29% in summer. They speculated that the spring–summer peak in thyrotoxicosis incidence in Britain may be causally related to the high milk iodine levels in winter–spring. In the United States, Park *et al.* (1981) estimated that dairy products contributed 38–56% of the total iodine intake of adults and 56–85% in infants and toddlers for 1974–1978. However, the Food and Drug Administration's Total Diet Study has shown steadily declining iodine intakes in the United States since 1982. In 1982–1983, the typical iodine intakes (excluding intakes from iodized salt) for men and women were 590 and 350 μg/day, respectively, while in 1985–1986, the typical

daily iodine intakes for men and women were 250 and 170 μg, respectively (Pennington *et al.*, 1989).

Most (80–90%) of the iodine in cow milk is in the inorganic form, mainly as iodide, and is located in the water-soluble fraction (Wheeler *et al.*, 1983; Miller *et al.*, 1975); 5–13% is bound to proteins through either covalent bonds or loose physical associations, with less than 0.1% bound to fat (Miller *et al.*, 1975; Murthy and Campbell, 1960). Iodide is rapidly and almost completely absorbed, but organically bound iodine is less well absorbed (NRC, 1989a).

XIII. MOLYBDENUM

Molybdenum is an essential component of several enzymes, including xanthine oxidase, aldehyde oxidase, and sulfite oxidase, where it occurs in the prosthetic group, molybopterin (Rajagopalan, 1988). It is not known whether the human requirement is for molybdenum per se or for molybopterin (or a precursor). Although molybdenum deficiency has been reported in a patient on long-term total parenteral nutrition therapy (Abumrad *et al.*, 1981), dietary deficiency of molybdenum has not been observed in humans (Rajagopalan, 1988). Recommended safe and adequate daily intakes of molybdenum are 15–30 μg for infants under 6 months old; 20–40 μg for infants 6–12 months old; 25–50, 30–75, and 50–150 μg for children 1–3, 4–6, and 7–10 years old, respectively; and 75–250 μg for adolescents and adults (NRC, 1989a).

The mean molybdenum content of cow milk is 50 μg/liter (Tsongas *et al.*, 1980), similar to values published earlier (42–73 μg/liter; Archibald, 1958). Milk molybdenum concentration has been shown to be dependent on dietary intake, increasing over fivefold when cows were supplemented with ammonium molybdate (Archibald, 1951). The molybdenum content of human milk has been reported to be strongly dependent on the stage of lactation, decreasing from 15 μg/liter on day 1 to 4.5 μg/liter on day 14 and to ~2 μg/liter at 1 month and thereafter (Casey and Neville, 1987).

All of the molybdenum present in cow milk is considered to be associated with xanthine oxidase (Hart *et al.*, 1967). However, when the molybdenum concentration in milk was increased by dietary supplementation, there was no corresponding increase in milk xanthine oxidase activity (Archibald, 1958; Hart *et al.*, 1967). Molybdenum is also associated with xanthine oxidase in the milk fat globule membrane in human milk, and human colostral xanthine oxidase has been purified and characterized (Zeise and Zikakis, 1987). The xanthine oxidase activity of human colostrum is about one-tenth that of cow milk (Oliver *et al.*, 1971).

XIV. CHROMIUM

Chromium is regarded as an essential nutrient for humans; the earliest detectable effect of deficiency is an impairment of glucose tolerance (Offenbacher and Pi-Sunyer, 1988). Chromium deficiency has only been unequivocally demonstrated in patients receiving long-term total parenteral nutrition therapy; these patients responded to intravenous trivalent chromium with improved glucose tolerance (Offenbacher and Pi-Sunyer, 1988). The exact requirements for chromium are unknown, but recommended safe and adequate daily intakes are 10–40 and 20–60 μg for infants aged 0–6 and 6–12 months, respectively; 20–80 and 30–120 μg for children aged 1–3 and 4–6 years, respectively; and 50–200 μg for children aged 7–10 years, adolescents, and adults (NRC, 1989a).

The mean chromium concentration in cow's milk has been reported as 2 μg/liter (range, 0.2–3.6 μg/liter) (Muzzarelli *et al.*, 1983), a value that is considerably lower than those reported earlier, e.g., 17 μg/liter (range, 5–50 μg/liter) (Renner, 1983). The mean chromium content of mature human milk is 0.27 μg/liter (Casey *et al.*, 1985), much lower than earlier reported values, e.g., 29 μg/liter (Murthy, 1974). The more recent values for chromium in milks are more reliable due to improved analytical methodology.

The chemical form of chromium in milk is unknown, although chromium in foods is generally in the trivalent state (NRC, 1989a). Recently, a 1500-Da chromium compound, containing aspartate, glutamate, glycine, and cysteine in the ratio 5:4:2:1, was isolated from bovine colostrum and shown to possess a biological activity similar to that of the glucose tolerance factor from yeast (Yamamoto *et al.*, 1988).

XV. COBALT

The only known function of cobalt in humans is its presence as an essential component of vitamin B_{12} (Smith, 1987). Because all vitamin B_{12} is derived from bacterial synthesis, inorganic cobalt can be considered essential for animal species that depend totally on their bacterial flora for their vitamin B_{12}. This is the case for ruminant animal species, in whom cobalt deficiency is well known. However, there is no evidence that the intake of cobalt is ever limiting in the human diet, and no RDA is necessary (NRC, 1989a).

The mean cobalt concentration in cow milk (0.5 μg/liter; range, 0.4–1.1 μg/liter) is higher than in mature human milk (0.1 μg/liter) (Smith, 1987). Dietary supplementation with cobalt increases the cobalt concentration in cow milk but does not increase the vitamin B_{12} content of milk unless the diet is cobalt deficient (Smith, 1987).

XVI. FLUORIDE

Fluoride accumulates in the hard tissues of the body (bones and teeth) (Krishnamachari, 1987), and although it is not strictly an essential element, it is regarded as a beneficial element for humans because of its protective role against dental caries (NRC, 1989a). Excessive intake of fluoride causes fluorosis, which causes mottling of teeth and affects bone health and kidney function (Krishnamachari, 1987).

The recommended safe and adequate daily intakes of fluoride are 0.1–0.5 mg for infants up to 6 months of age, 0.2–1.0 mg for infants aged 6–12 months, 0.5–1.5 mg for children aged 1–3 years, 1.0–2.5 mg for children aged 4–6 years, 1.5–2.5 mg for children aged 7–10 years and adolescents, and 1.5–4.0 mg for adults (NRC, 1989a).

The mean fluoride content of mature human milk is 16 μg/liter (AAP, 1985), but reported mean values range from 5 to 25 μg/liter (Esala *et al.*, 1982; Krishnamachari, 1987; Spark *et al.*, 1983), reflecting maternal intake. The mean fluoride content of cow milk is 20 μg/liter (range, 10–140 μg/liter) (Taves, 1983; Wheeler *et al.*, 1988). About 46–64% of the fluoride in cow milk occurs as free fluoride ions, with the remainder bound to proteins (Esala *et al.*, 1982).

Fluoride intake is low in infants consuming breast milk or concentrated or powdered formulas prepared with nonfluoridated water (NRC, 1989a), and these infants should receive fluoride supplements (AAP, 1985). Spak *et al.* (1982) found that absorption of fluoride from sodium fluoride added to milk or infant formula in young adults was 65–70%.

XVII. ARSENIC, NICKEL, SILICON, and BORON

There is substantial evidence to establish the essentiality of arsenic, nickel, silicon, and boron in animals, and it is likely that these trace elements are also essential for humans (Nielsen, 1988). However, the nutritional functions of these elements are still unclear and there are no reliable data on which to base estimates of human requirements (NRC, 1989a). Reported concentrations of these elements in human and cow's milks are presented in Table I.

XVIII. SUMMARY AND CONCLUSIONS

The nutritional roles, requirements, and metabolism and the quantitative relationship between dietary intakes and health for a number of the minerals and

trace elements have been more clearly defined in recent years, but there are still considerable deficiencies in our understanding of these issues, e.g., the significance of calcium in the etiology and treatment of osteoporosis and hypertension.

Reliable information is now available on the content, and the principal factors affecting it, of most of the minerals and trace elements in human and cow's milks. However, for some of the trace elements, there is still a wide variation in reported values in the literature, which is due, at least in part, to analytical difficulties. The contribution of cow milk and milk products to the diet in Western countries is significant for sodium, potassium, chloride, calcium, phosphorus, zinc, and iodine. Iodine is the only trace element for which there has been any suggestion of excessive amounts in cow milk. However, there is evidence of a decline in milk iodine concentrations in the United States in recent years, although the situation in other countries less clear.

Breast milk usually has adequate mineral and trace element contents for feeding full-term infants, with the exceptions of fluoride, for which supplementation of infants is recommended, and of selenium in some countries, such as Finland and New Zealand, where maternal intakes are low. However, breast milk selenium contents have increased in these countries in recent years due to increased maternal selenium intakes.

The concentrations of minerals and trace elements in infant formulas for full-term infants are generally higher than in human milk, and all appear to be more than adequate, with the possible exception of selenium, which may need to be increased in some formulas. Considerable changes in the mineral and trace element contents of formulas have been instituted in recent years in the light of improved knowledge of infant requirements.

While the chemical forms of the macrominerals and some of the trace elements (iron, zinc, copper, and manganese) in milks are fairly well defined, the forms of many of the trace elements are unknown.

Sodium, potassium, chloride, and iodine are believed to be almost totally absorbed from milks and infant formulas. The bioavailability of some other minerals and trace elements (e.g., calcium, iron, and zinc) in human milk appears to be very high (generally higher than in cow milk), but the basis for this remains unclear. There is little information on the bioavailability of magnesium, copper, manganese, selenium, fluoride, or other trace elements in milks and formulas. Factors affecting the bioavailability of minerals and trace elements in milks are still poorly understood, and the role of possible enhancers (e.g., lactose, ascorbate, citrate, phosphopeptides, and lactoferrin) or inhibitors (e.g., proteins, calcium, and phosphate) of mineral and trace element absorption in milks remains unclear. However, milks do not appear to contain substances that are strongly inhibitory to mineral and trace element absorption, such as phytate or the polyphenols found in plant foods.

XIX. RESEARCH NEEDS

There is a need for continued monitoring of the iodine concentrations in cow milk and, where necessary, to reduce them by improved farm management practices. Modern analytical methodology should be applied more widely to the quantitation of trace elements in milks and infant formulas. Speciation studies are required in order to characterize the chemical forms of a number of trace elements in milks.

Ensuring the safety and adequacy of existing levels of minerals and trace elements in infant formulas requires further study, and optimal levels need to be defined more precisely. For example, can supplementation levels of iron be reduced without increasing the risk of deficiency? What is the best means of increasing selenium content? Also, the ratios of the concentrations of minerals and trace elements in formulas need to be examined due to possible interactions affecting absorption and/or utilization (Lonnerdal, 1989b).

Further studies are needed on the bioavailability of minerals and trace elements in milks and infant formulas and the basis of the apparently high bioavailability of some of these in human milk needs clarification. The role of potential enhancers (e.g., lactose, ascorbate, citrate, phosphopeptides, and lactoferrin) or inhibitors (e.g., proteins, calcium, and phosphate) of mineral and trace element absorption in milks needs to be defined.

There is a need for improved animal models for the study of factors that affect bioavailability, and these should be used to complement studies in humans. There is also a need for standardization of methods for assessment of bioavailability in humans. The recent development of stable isotope techniques holds considerable promise for improvement in our knowledge of bioavailability of a number of minerals and trace elements, particularly in infants and children. Studies are required on how the consumption of milk with other foods affects the bioavailability of minerals and trace elements in whole meals.

However, an understanding of the nutritional significance of minerals and trace elements in milk will also benefit from improvement in our knowledge of fundamental aspects of minerals and trace elements, such as their nutritional roles, requirements and metabolism, and the quantitative relationship between dietary intakes and health.

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ADVANCES IN FOOD AND NUTRITION RESEARCH, VOL. 36

DIETARY FATTY ACIDS, LIPOPROTEINS, AND CARDIOVASCULAR DISEASE

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I. INTRODUCTION

The role of dietary fatty acids, with respect to both type and amount, in determining plasma lipoprotein levels and cardiovascular disease risk has been an area of intense basic and applied investigation for several decades. There is little question that the quality and quantity of dietary fatty acids play a significant role in determining plasma lipid levels through effects on the metabolism of both atherogenic and antiatherogenic plasma lipoproteins. Numerous intervention studies have shown that modifications in the type and amount of dietary fatty acids affect plasma lipoprotein levels and, in theory, the cardiovascular disease (CVD) risk profile. Conventional wisdom held that intake of saturated fatty acids (SFA) increased plasma cholesterol levels whereas polyunsaturated fatty acids (PUFA) in the diet lowered plasma total cholesterol concentrations.

Detailed studies of the effects of specific types of fatty acids have shown that this oversimplification of the response of plasma cholesterol to dietary fat fails to account for differential effects on specific lipoprotein fractions (and subfractions) or for unique effects of fatty acid chain lengths and degree of unsaturation on these responses. It is now clear that *n*-3 PUFA (where *n-x* corresponds to ω-*x* unsaturated fatty acids) exert different effects than the *n*-6 series of these fatty acids, that not all SFA increase plasma cholesterol levels, and that different dietary fatty acids can have unique effects on the metabolism of specific plasma lipoproteins: chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). This review evaluates the current status of our understanding of the effects of dietary fatty acids and mechanisms by they mediate changes in plasma lipoprotein levels as related to

CVD risk, and the potential benefits of changes in the fatty acid composition of the diet as a modality to reduce CVD incidence in the population.

Two important considerations involved in any evaluation of the role of dietary fatty acids in determining plasma lipoprotein levels are an understanding of cholesterol and lipoprotein metabolism and the critical evaluation of the design and interpretation of dietary studies, whether these are animal model studies or clinical investigations in a human population.

A. DESIGN OF DIETARY STUDIES

Evaluation of the design and interpretation of dietary studies is a complex process in that, while diets are easy to formulate and test, a diet's relationship to patterns of human nutrient consumption may have little relevance in the real world. Humans do not consume liquid formula diets consisting of purified components; human diets rarely consist of a single class of fatty acids or extremes of calories from either SFA, monounsaturated fatty acids (MUFA), or PUFA; and pharmacological levels of dietary cholesterol far exceeding endogenous cholesterol synthesis are not part of a typical American diet. It is important to note that many of the nonphysiological extremes utilized to assure a measurable effect of dietary fatty acids on plasma lipoprotein levels do not *a priori* represent the responses obtained from intake of moderate, balanced diets. In many cases it is clearly inappropriate to extrapolate data on the effects of dietary excesses to the more modest effects of either the current American diet or to recommended changes in dietary fat, when considered in relationship to moderate diets consisting of a variety of foods.

A consideration in evaluating animal studies of dietary fat effects on lipoprotein metabolism is that humans have a strikingly different plasma lipoprotein profile (high LDL, low HDL) compared to animal model systems (high HDL, low LDL) used in the majority of studies. Many animal studies also suffer from the fact that, since dietary fat has a higher caloric density (9 kcal/g) than carbohydrates (4 kcal/g), addition of fat to a test diet often fails to account for corresponding changes in the caloric density of the diet. In many published reports, differences in the caloric densities of the test diets have resulted in variations not only in dietary fat intake but also in the intake of protein, fiber, vitamins, and minerals, all of which can exert their own effects on plasma lipoprotein levels and metabolism. Only by appropriate design of test diets can the specific effects of modifications in dietary fat quality and quantity on lipoprotein levels and metabolism be determined. Unfortunately, the literature is replete with examples of poorly designed dietary fat studies and such studies must be viewed with appropriate skepticism. Always keep in mind that any evaluation of the evidence regarding dietary fatty acid effects on lipoprotein metabolism and atherogenesis from a single study should be considered with respect to the experimental animal

model chosen; the appropriateness of the dietary design; the relevance of the interventions, whether physiological or pharmacological; and appropriate recognition of interpretation limitations of data from animal model studies as related to human health and disease incidence.

There is one final cautionary note for any evaluation of data on dietary effects on plasma lipids, lipoproteins, and their metabolism, especially of clinical studies. In many cases conclusions are based on the failure to detect significant differences between diet A and diet B, yet rarely are the data analyzed to provide an estimate of the power of the study. In other words, what was the chance of finding a significant effect considering the number of subjects studied and the variance of the measured parameters? The literature is filled with studies that, due to the large degree of metabolic heterogeneity among patients, the physiological variability within patients, and the relatively small numbers of patients studied, had only a 40–60% chance, or less, of detecting a significant difference even if one in fact existed. Rarely are studies analyzed to determine the potential for β-error in the use of statistical analysis to derive conclusions from the data. Analysis of data to express the reliability of either rejecting or accepting the null hypothesis should be provided, not only when the hypothesis is rejected (the *P* values presented in most published studies), but also when the null hypothesis is accepted by providing values for the power of the study. Without both statistical considerations it is difficult for the reader to compare the relative merits of a study that concludes no effect of an intervention versus another study concluding that a significant effect exists.

B. PLASMA LIPOPROTEINS

Plasma lipoproteins are complexes of lipids and proteins that facilitate lipid transport and metabolism. Lipoproteins consist of a hydrophobic core of nonpolar lipids—triacylglycerols and esterified cholesterol—with a hydrophilic coat of phospholipid, unesterified cholesterol, and apolipoproteins (Table I). Lipoprotein transport and metabolism are in large part regulated by the apolipoproteins on the surface of the particles, which serve as structural components, ligands for cell receptors, and cofactors for enzymes involved in lipoprotein metabolism (Table II). There are four major classes of lipoproteins, the classes are based on density and on lipid and apolipoprotein (apo) composition: chylomicrons, VLDL, LDL, and HDL. These general categories include various subclasses of lipoproteins, such as intermediate density lipoprotein (IDL), a lipoprotein of density and composition between VLDL and LDL, and two major classes of HDL, HDL_2 and HDL_3. Within each of the major and minor lipoprotein categories there exists a large degree of size heterogeneity and genetically regulated interindividual variability in the distribution of the particles (Fisher *et al.* 1989). While there are significant differences in size and composition of the various lipoproteins,

TABLE I

CHARACTERISTICS AND FUNCTION OF THE MAJOR PLASMA LIPOPROTEINS[a]

Characteristic	Classes of plasma lipoproteins					
	Chylomicrons	VLDLs	IDLs	LDLs	HDL_2	HDL_3
Density (g/ml)	<0.95	0.95–1.006	1.006–1.019	1.019–1.063	1.063–1.12	1.12–1.21
Diameter (nm)	75–1200	30–80	15–35	18–25	10–20	7.5–10
Molecular weight	400×10^6	$10–80 \times 10^6$	$5–10 \times 10^6$	2.3×10^6	$1.7–3.6 \times 10^6$	
Origin	Intestine	Liver	VLDLs	VLDLs/IDLs/liver	Intestine and liver	
Electrophoretic mobility	Origin	Pre-β	Pre-β to β	β	α	
Physiological role	Exogenous fat transport	Endogenous fat transport	LDL precursor	Cholesterol transport	Reverse cholesterol transport	
Composition (%)						
Triacylglycerol	86	52	28	10	10	5
Cholesteryl ester	3	14	30	38	21	14
Free cholesterol	1	7	8	8	7	3
Phospholipid	8	18	23	22	29	19
Protein	2	8	11	21	33	57

[a]Information derived in part from material in Kris-Etherton *et al.* (1988) and Ginsberg (1990).

TABLE II

APOPROTEINS OF HUMAN PLASMA LIPOPORTEINS[a]

Apolipoprotein	Molecular weight	Plasma concentration (g/liter)	Lipoprotein distribution	Function
Apo-A-I	28,000	1.0–1.2	Chylomicrons, HDL	LCAT activation
ApoA-II	17,000	0.3–0.5	Chylomicrons, HDL	Unknown
ApoA-IV	46,000	0.15–0.16	Chylomicrons, HDL	Unknown
ApoB-48	264,000	0.03–0.05	Chylomicrons	Structural
ApoB-100	512,000	0.7–1.0	VLDL, IDL, LDL	Receptor ligand
ApoC-I	7000	0.04–0.06	Chylomicrons, VLDL, HDL	—
ApoC-II	9000	0.03–0.05	Chylomicrons, VLDL, HDL	LPL activation
ApoC-III	9000	0.12–0.14	Chylomicrons, VLDL, HDL	LPL inhibition
ApoD	33,000	0.06–0.07	HDL	Cholesteryl ester transfer (?)
ApoE	38,000	0.03–0.05	Chylomicrons, VLDL, IDL, HDL	Receptor ligand (remnant and LDL)

[a]Information derived in part from material in Kris-Etherton *et al.* (1988), Fisher *et al.* (1989), and Ginsberg (1990).

they all share the same spherical structure of a hydrophobic core and a hydrophilic surface, allowing transport of insoluble lipids through the plasma compartment. The different plasma lipoproteins have specific roles in facilitating exogenous and endogenous lipid transport and each makes its own contribution to an individual's CVD risk profile (Ginsberg, 1990).

1. Chylomicrons

The large, triacylglycerol-rich chylomicrons transport exogenous fatty acids from the intestine to peripheral tissues and the liver. Chylomicrons are produced in the intestine and transport dietary fat and dietary and biliary cholesterol. Nascent chylomicrons contain apo-48, apoA-I, and apoA-IV, and following transport via the lymphatics to the blood, acquire apoCs and apoE from circulating HDL. Chylomicron metabolism occurs initially in peripheral tissues, where lipoprotein lipase (PL), a capillary endothelial cell surface enzyme that is activated by apoC-II on the chylomicrons, hydrolyzes the triacylglycerol core, releasing free fatty acids for tissue uptake. As the chylomicron loses its triacylglycerol core, the particle becomes smaller and is termed a chylomicron remnant. During this process, surface lipids and apoproteins are released to plasma HDL. The chylomicron remnant is rapidly removed from the circulation via specific receptors on hepatic membranes and is catabolized in the liver.

2. *VLDL*

Endogenous fat transport is accomplished via the synthesis, secretion, and catabolism of VLDL. The liver produces and secretes triacylglycerol-rich VLDL particles that contain apoB-100, apoCs (I, II, and III), and apoE. Plasma VLDL receive additional cholesteryl esters from HDL by the action of cholesteryl ester transfer protein (CETP), which also acts to transfer cholesteryl ester to LDL. As with chylomicrons, VLDL triacylglycerols undergo hydrolysis by endothelial cell LPL, which are activated by apoC-II. The VLDL remnant (IDL) can undergo additional lipolytic removal of triacylglycerols and loss of apoproteins to eventually form LDL. Plasma VLDL can undergo a number of metabolic fates along this catabolic pathway: (1) up to 50% of the newly secreted VLDL can be cleared by direct hepatic removal (Packard *et al.*, 1984) via the LDL receptor, and possibly via the chylomicron remnant and/or the LDL receptor-related protein receptors (Kowal *et al.* 1989); (2) catabolism to IDL, which also can be cleared by the liver through LDL receptor-mediated mechanisms; or (3) continued intravascular processing to eventually form LDL. As will be discussed in more detail later, dietary fatty acids play a role in determining which metabolic sequence VLDL will undergo.

3. *LDL*

The major cholesterol-carrying lipoprotein in humans is LDL, with a lipid core almost entirely of cholesteryl ester and a single apoprotein, apoB-100. Approximately 75% of plasma LDL is cleared by the liver (Bilheimer *et al.*, 1984), and each day 30–40% of the LDL pool is turned over. The majority (60–80%) of LDL catabolism occurs via LDL receptor-mediated processes with the remainder catabolized by receptor-independent mechanisms (Kesaniemi *et al.*, 1983). Circulating levels of LDL cholesterol are determined by the balance between LDL production (mg/kg/day) and LDL removal (fractional catabolic rate in pools/day). A key regulator of LDL catabolism is expression and activity of the LDL (apoB/E) receptor, which accounts for the majority of LDL catabolism (Brown and Goldstein, 1986). In terms of CVD risk, it is clear that an elevated plasma LDL cholesterol level constitutes a major CVD risk factor (Stamler *et al.*, 1986), and lowering an elevated LDL cholesterol level is the primary objective of dietary interventions to reduce CVD incidence (The Expert Panel, 1988).

4. *HDL*

HDL is synthesized by the liver and intestine and is secreted as disk-shaped particles containing unesterified cholesterol, phospholipid, apoA-I, apoE, and apoCs. The hydrolysis of chylomicrons and VLDL by LPL results in a net transfer of phospholipids and unesterified cholesterol to HDL. In addition, HDL obtain

free cholesterol from cell membranes, in theory as part of the "reverse cholesterol transport system." The HDL-associated enzyme lecithin:cholesterol acyltransferase (LCAT) is activated by apoA-I and catalyzes the synthesis of cholesteryl ester from phospholipids and free cholesterol; the hydrophobic cholesteryl ester then moves into the core of the HDL particle, allowing for influx of more phospholipid and unesterified cholesterol (Tall, 1990). In humans and some animal species the majority of cholesteryl esters formed in HDL are transferred to apoB-containing lipoproteins in exchange for triacylglycerols (Tall, 1990). This process of lipid and apoprotein exchange between HDL and the triacylglycerol-rich lipoproteins partially explains why plasma HDL cholesterol levels are related to the metabolism and intravascular processing of chylomicrons and VLDL.

HDL particles undergo a cycle of enlargement (lipid influx and LCAT catalysis of cholesteryl ester formation), exchange (cholesteryl ester for triacylglycerol), and contraction (triacylglycerol and phospholipid hydrolysis by hepatic triacylglycerol lipase). During this cycle the smaller HDL_3 enlarges to become the cholesteryl ester-rich HDL_2 particle, which subsequently undergoes conversion back to an HDL_3 particle. The major portion of plasma HDL is usually HDL_3 and variations in HDL levels usually reflect different amounts of this HDL subfraction. HDL cholesteryl esters are turned over at a rate 10–40 times that of the major HDL apoproteins due to exchange with triacylglycerol-rich lipoproteins and to selective removal of HDL cholesteryl esters by the liver and other tissues (Pittman *et al.*, 1987). Specific HDL-binding proteins have been described (Graham and Oram, 1987), but their role in HDL metabolism remains to be determined.

Epidemiological and clinical studies have demonstrated an inverse relationship between plasma HDL levels and the incidence of coronary artery disease (T. Gordon *et al.*, 1977; D. J. Gordon *et al.*, 1989). Data from drug intervention trials suggest that effects on HDL levels alter the CVD risk profile of patients, independent of effects on LDL cholesterol levels (Tall, 1990); however, to date there have been no clinical trials directly testing the coronary artery disease risk reduction benefits for elevating plasma HDL levels.

C. PLASMA LIPOPROTEINS AND CARDIOVASCULAR DISEASE RISK

There is little debate that elevated plasma cholesterol levels are positively related to increased coronary heart disease (CHD) risk (Stamler *et al.*, 1986; The Expert Panel, 1988). Plasma concentrations of both the lipoproteins and apolipoproteins are strong predictors of cardiovascular disease risk: plasma total cholesterol, LDL cholesterol, and apoB levels are positively correlated with risk while plasma HDL cholesterol (primarily HDL_2) (Miller, 1987) and apoA-I levels are negatively correlated with risk. The National Cholesterol Education Pro-

gram's Adult Treatment Panel (The Expert Panel, 1988) has classified CHD risk as average when plasma total cholesterol levels are less than 200 mg/dl (<5.17 mmol/liter), moderate for values between 200 and 240 mg/dl (5.17–6.21 mmol/liter), and high for total cholesterol levels greater than 240 mg/dl (>6.21 mmol/liter). (To convert mg/dl cholesterol to mmol/liter, multiple by 0.02586.) Based on plasma LDL cholesterol concentrations, CHD risk is classified as moderate for individuals with levels of 130 to 159 mg/dl, and levels of greater than 160 mg/dl represent high risk. HDL cholesterol values of less than 35 mg/dl are also considered to represent an increased risk for CHD.

The ratio of total cholesterol to HDL cholesterol, or more specifically the LDL cholesterol to HDL cholesterol ratio, is a significant indicator of CHD risk (Gordon *et al.*, 1977; Castelli *et al.*, 1986) and is an important consideration in evaluating dietary interventions to lower CVD risk profiles. There is increasing evidence that plasma apolipoprotein levels may be better discriminators of CHD risk than plasma lipid or lipoprotein levels (Reinhart *et al.*, 1990), with the ratio of apoA-I to apoB being a significant discriminator of CVD risk; levels of apoB are elevated and apoA-I and apoA-II are reduced in CHD subjects. As discussed below, changes in dietary fat saturation and amount modify plasma lipid levels by alterations in both the lipid content of the lipoproteins and the number of lipoprotein particles. Data regarding effects of dietary fatty acid chain length, unsaturation, and amount on plasma apolipoprotein levels are limited at this time, representing an important area of investigation in evaluating the relative effectiveness of changes in the amount and type of dietary fat in CVD risk reduction.

II. EPIDEMIOLOGICAL EVIDENCE OF A RELATIONSHIP BETWEEN DIETARY FAT SATURATION AND CARDIOVASCULAR DISEASE

Both international and within-population epidemiological studies indicate a positive relationship between plasma cholesterol levels and CHD mortality (Keys *et al.*, 1986; Stamler *et al.*, 1986; The Expert Panel, 1988). A consistent finding of most international epidemiological studies has been the observation of a positive relationship between calories from SFA in the diet, plasma cholesterol levels, and rates of CHD mortality. The Seven Countries Study provides some of the strongest epidemiological evidence for a relationship between dietary SFA and CHD mortality (Keys *et al.*, 1986). The 15-year mortality data for 11,579 middle-aged men indicated a positive correlation between SFA calories and CHD mortality rates (Fig. 1), and with rates of total mortality (Keys *et al.*, 1986). The 15-year death rate from CHD was also related to the MUFA:SFA ratio (Fig. 1), with 44% of the variance in CHD death accounted for by this single variable. Inclusion of age, body mass index (an indicator of excess body weight), systolic blood pressure, serum cholesterol, number of cigarettes smoked daily, and the

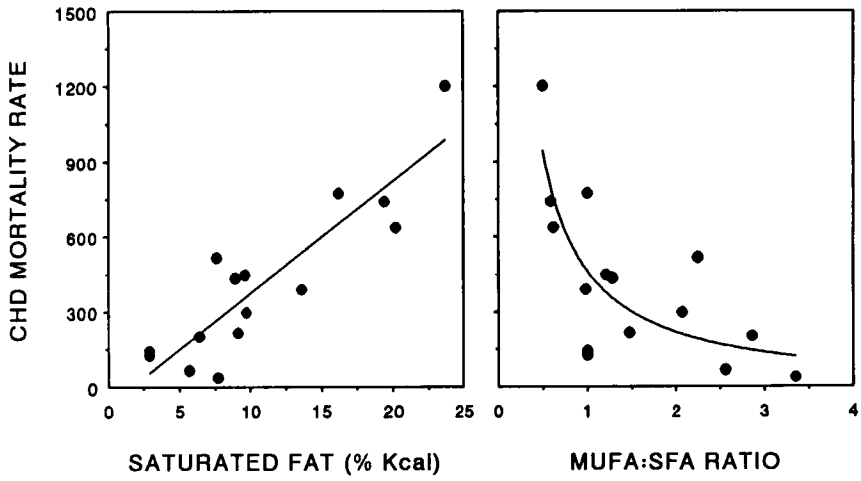

FIG. 1. Epidemiological data comparing percentage of calories from fat with coronary heart disease mortality. Relationships between calories (%) from saturated fatty acids (left) and ratio of calories from monounsaturated and saturated fatty acids (MUFA:SFA) (right) to coronary heart disease (CHD) mortality rates in the Seven Countries Study. (After Keys *et al.*, 1986.)

dietary MUFA:SFA ratio in the multiple regression analysis indicated that 96% of the variance in CHD mortality between the study populations could be accounted for by these inherent and life-style factors (Keys *et al.*, 1986).

In a comparable analysis of dietary and mortality data from 18 countries, Hegsted and Ausman (1988) also reported significant correlations between percentage of calories from SFA and CHD mortality rates. While both the Keys *et al.* (1986) and Hegsted and Ausman (1988) studies demonstrated a significant positive relationship between percentage of calories from SFA and CHD mortality rates, neither of these international studies found a negative relationship between PUFA calories and CHD mortality. It is important to note that interpretations of these two analyses of international data on the relationship between SFA intake and CHD mortality rates differ in that the SFA effect is significantly more pronounced in the Seven Countries Study data base than in the 18-country analysis.

Numerous epidemiological studies have reported a relationship between saturated fat calories and CHD mortality. These include the Japan–Honolulu–San Francisco Study (Kagan *et al.*, 1974), the Honolulu Heart Program (Kato *et al.*, 1973; McGee *et al.*, 1985), the Western Electric Study (Shekelle *et al.*, 1981), the Ireland–Boston Study (Kushi *et al.*, 1985), and the Zutphen Study (Kromhout and de Lezenne Coulander, 1984). Based on the congruency of the epidemiological data it is only reasonable to conclude that a consistent and significant relationship between dietary intake of SFA and CHD mortality exists in almost

all populations. Whether this is due to a direct cause-and-effect relationship cannot be determined from the epidemiological data. While the data implicate saturated fats as having a role in CHD mortality, the epidemiological data do not consistently support the inference that PUFA or MUFA in the diet are protective against CHD.

A recent report from the Framingham Study (Posner *et al.*, 1991) indicates that in young men aged 45 to 55 years there was a significant positive association between the 16-year incidence of CHD and the proportion of dietary calories from total fat and MUFA, even after adjustment for other CHD risk factors. The finding of a significant relationship between CHD incidence and dietary fat intake after adjustment for other risk factors raises the possibility that diet may exert an effect on CHD incidence independent of its effect on plasma lipid levels. Interestingly, the data analysis could not demonstrate a significant association between dietary fat calories and CHD morbidity or mortality in men older than 55 years or a protective role regarding CHD incidence for PUFA in the diet. The relationship between SFA calories and CHD incidence was borderline significant in the younger age group and not significant in the older population. While epidemiological studies have not been able to demonstrate a relationship between PUFA intake and reduced CHD incidence, there is evidence to suggest that intake of *n*-3 polyunsaturated fish oils, when consumed as fish, can significantly reduce CHD incidence in a population (Kromhout *et al.*, 1985; Shekelle *et al.*, 1985).

III. DIETARY FATTY ACIDS

For the purposes of this review, dietary fatty acids will be considered as four classes: SFA, MUFA, *n*-6 PUFA, and *n*-3 PUFA. While this is the classical grouping of dietary fatty acids used to report the findings of metabolic studies, it will be seen that the metabolic responses of the plasma lipoproteins to dietary fat variations between and within each classification can vary depending on fatty acid chain length and degree of unsaturation.

A. SATURATED FATTY ACIDS

SFA vary in chain length from 4 to 18 carbons and the major saturated fatty acids in the diet are lauric (C12:0), myristic (C14:0), palmitic (C16:0), and stearic (C18:0) acids. The major dietary sources of saturated fatty acids are animal products (meats and dairy products), tropical oils (coconut, palm, and palm kernel oils) added to processed foods, and hydrogenated vegetable oils. The intake of saturated fatty acids in the American diet averages 13% of total calories; and the major SFA is palmitic acid (Fig. 2). It has been recommended

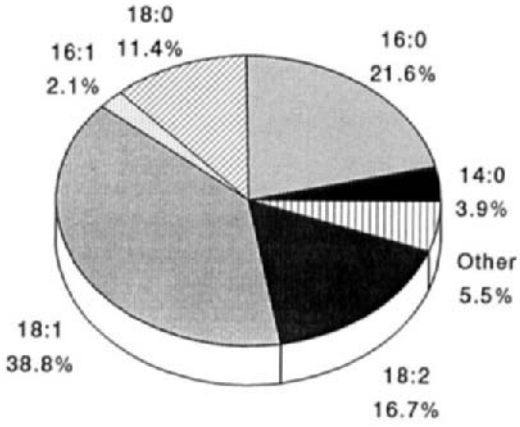

FIG. 2. Fatty acid composition of a typical American diet. Contribution of specific fatty acids in a simulated American diet presented as percentage of total fat. (After Ahrens and Boucher, 1978.)

by various health agencies that SFA intake be reduced to less than 10% of total calories (The Expert Panel, 1988; Committee on Diet and Health, 1989; *Surgeon General's Report,* 1988) based on epidemiological and metabolic evidence that high levels of SFA in the diet are related to an increase in plasma cholesterol levels and a higher CVD incidence.

B. MONOUNSATURATED FATTY ACIDS

The primary MUFA in the diet are palmitoleic (C16:1, *n*-9) and oleic (C18:1, *n*-9) acids; however, for practical purposes this class of fatty acids is represented by oleic acid (Fig. 2), which is found in olive oil, rapeseed oil (canola oil), cocoa butter, and beef. In addition, humans have the ability to synthesize MUFA from SFA. A typical diet contains 15–16% of its calories from MUFA and the dietary recommendation has been to reduce MUFA intake to 10% of calories. Conventional wisdom held that MUFA had a neutral effect on plasma cholesterol levels, but, as will be seen later, recent studies have shown the MUFA, when substituted for SFA in the diet, effectively lower plasma LDL cholesterol concentrations and could play an important role in dietary fat modifications to lower plasma cholesterol levels (Grundy, 1989).

During the process of hydrogenation of PUFA vegetable oils, considerable quantities of trans MUFA can be produced. The major trans-unsaturated isomer is elaidic acid (C18:1, trans *n*-9) as well as other isomers. While a number of studies suggested that trans MUFA had a neutral affect on plasma cholesterol levels similar to oleic acid, recent studies indicate that trans MUFA alter plasma lipoprotein levels toward a more atherogenic profile.

C. POLYUNSATURATED FATTY ACIDS

There are two major classes of PUFA: ω-3 (*n*-3) fatty acids, such as eicosapentaenoic (C20:5, *n*-3) and docosahexaenoic (C22:6, *n*-3) acids, found in fish oils and as minor constituents of some vegetable oils; and ω-6 (*n*-6), fatty acids, including the essential linoleic acid (C18:2, *n*-6), found in vegetable oils such as corn, cottonseed, and soybean oils. In the current American diet, calories from PUFA average 5–7%, although this value has been increasing over the years due to changes in the eating habits of the public (Fig. 3) (Stephen and Wald, 1990). Dietary recommendations indicate that PUFA in the diet should approach 10% of total calories, based on the plasma LDL cholesterol-lowering effect of *n*-6 PUFA (The Expert Panel, 1988; Committee on Diet and Health, 1989; *Surgeon General's Report,* 1988).

There have been no specific dietary recommendations regarding the intake of *n*-3 PUFA; however, there have been cautions raised regarding intake of fish oil supplements, which are advertised as an approach to lowering plasma cholesterol levels and CVD risk. As discussed below, the effects of *n*-3 PUFA differ from those observed for the *n*-6 series and their potential plasma lipid-lowering effect in part depends on the hyperlipidemia expressed by the individual.

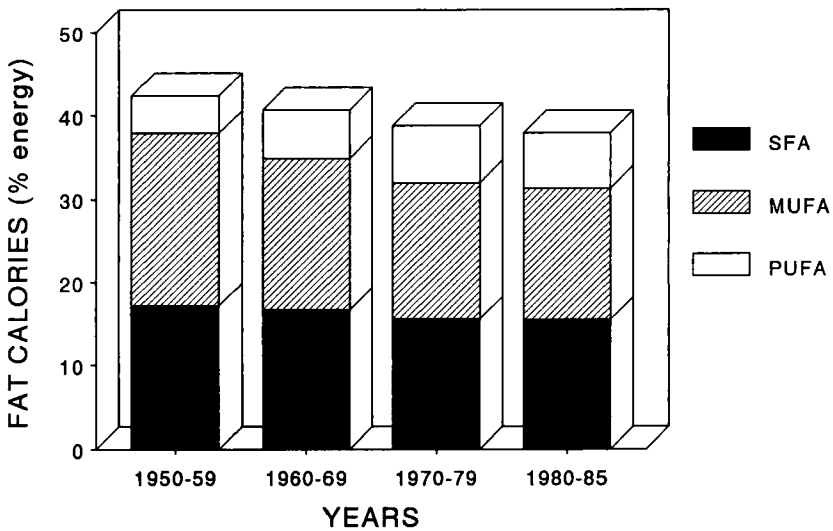

FIG. 3. Trends in dietary fat intake in the United States: 1950–1985. Data for percentage of dietary calories from saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) in the American diet between 1950 and 1985. (After Stephen and Wald, 1990.)

D. FATTY ACIDS OF COMMONLY USED DIETARY FATS

Presented in Table III are the fatty acid compositions of the dietary fats commonly used for studies of fatty acid effects on lipids and lipoproteins. These dietary fats exhibit a wide range of fatty acid types and varying ratios of PUFA:MUFA:SFA (P:M:S). What is important to note is that even though some dietary fats have the same P:S ratio, they contain differing amounts of MUFA and, in theory, would have significantly different effects on plasma lipoprotein concentrations. Fats such as cocoa butter and coconut oil are highly saturated (P:S ratio 0.02–0.05), but while coconut oil has a high content of lauric acid (C12:0) and few MUFA, cocoa butter contains mostly palmitic (C16:0) and stearic (C18:0) acids and is relatively rich in oleic acid (C18:1). The P:M:S ratios for cocoa butter and coconut oil are 0.05:0.55:1.0 and 0.02:0.07:1.0, respectively. While both fats have a low P:S ratio, the actual amounts of SFA and PUFA in each fat differ and would be predicted to have strikingly different effects on the plasma lipoprotein levels; the significance of such differences becomes even more important when considering that stearic acid, a major fatty acid found in cocoa butter, is not a typical SFA in that it does not raise plasma cholesterol levels in humans, as found for other SFA.

In addition to differences in the content of MUFA and stearic acid, some fats, such as butter oil, contain short-chain fatty acids (6 to 10 carbons) that are absorbed directly via the portal vein and are not transported through the bloodstream to the liver by chylomicrons. Thus, intake of short- and medium-chain fatty acids would be predicted to have different effects on the intravascular processing and interconversions of the lipoproteins compared to fats containing only long-chain fatty acids. Table III is presented so that the reader has a reference when comparing the relative effects of different dietary fats and oils on plasma lipids and lipoproteins in the studies discussed in this review.

IV. PLASMA LIPID/LIPOPROTEIN RESPONSES TO SATURATED FATTY ACIDS

Numerous epidemiological studies have demonstrated a positive relationship between the percentage of calories obtained from SFA in the diet and rates of CHD between and within populations (Keys *et al.*, 1986; Hegsted and Ausman, 1988; Kromhout and de Lezenne Coulander, 1984). As a class of fatty acids, the evidence clearly shows that intake of SFA increases plasma LDL cholesterol levels (McNamara, 1987; Grundy and Denke, 1990; Goodnight *et al.*, 1982; Goldberg and Schonfeld, 1985) as compared to intake of calories from either MUFA or PUFA, or of carbohydrates. These comparisons and the mechanisms involved will be discussed in detail later.

TABLE III

FATTY ACID COMPOSITION OF COMMONLY USED FATS IN STUDIES OF DIETARY FATTY ACID EFFECTS ON PLASMA LIPOPROTEINS

	Fatty acids (%)													
Fat	Beef tallow	Butter oil	Lard	Cocoa butter	Coconut	Corn oil	Cotton seed	Olive oil	Palm oil	Palm kernel	Rape seed	Safflower	Soybean	Sunflower
Total SFA	**49.8**	**61.9**	**39.2**	**59.78**	**86.5**	**12.7**	**25.9**	**13.5**	**49.3**	**81.4**	**6.8**	**9.1**	**14.4**	**10.3**
C8:0	—	1.1	—	—	7.5	—	—	—	—	3.3	—	—	—	—
C10:0	—	2.5	0.1	—	6.0	—	—	—	—	3.7	—	—	—	—
C12:0	0.9	2.8	0.2	—	44.6	—	—	—	0.1	47.0	—	—	—	—
C14:0	3.7	10.0	1.3	0.1	16.8	—	0.8	—	1.0	16.4	—	0.1	0.1	—
C16:0	24.9	26.2	23.8	25.4	8.2	10.9	22.7	11.0	43.5	8.1	4.8	6.2	10.3	5.9
C18:0	18.9	12.1	13.5	33.2	2.8	1.8	2.3	2.2	4.3	2.8	1.6	2.2	3.8	4.5
Total MUFA	**41.8**	**28.7**	**45.1**	**32.9**	**5.8**	**24.2**	**17.8**	**73.7**	**37.0**	**11.4**	**55.5**	**12.1**	**23.3**	**19.5**
C16:1	4.2	2.2	2.7	0.2	—	—	0.8	0.8	0.3	—	0.5	0.4	0.2	—
C18;1	36.0	25.0	41.2	32.6	5.8	24.2	17.0	72.5	36.6	11.4	53.8	11.7	22.8	19.5
C20:1	0.3	—	1.0	—	—	—	—	0.3	0.1	—	0.1	—	0.2	—
Total PUFA	**4.0**	**3.7**	**11.2**	**3.0**	**1.8**	**58.7**	**51.9**	**8.4**	**9.3**	**1.6**	**33.3**	**74.5**	**57.9**	**65.7**
C18:2	3.1	2.3	10.2	2.8	1.8	58.0	51.5	7.9	9.1	1.6	22.1	74.1	51.0	65.7
C18:3	0.6	1.5	1.0	0.1	—	0.7	0.2	0.6	0.2	—	11.1	0.4	6.8	—
Ratio[a]														
P	0.08	0.06	0.29	0.05	0.02	4.62	2.00	0.62	0.19	0.02	4.90	8.19	4.02	6.38
M	0.84	0.46	1.15	0.55	0.07	1.91	0.69	5.46	0.75	0.14	8.16	1.33	1.62	1.86
S	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

[a]Ratios of polyunsaturated (P), monounsaturated (M), and saturated (S) fatty acids.

It should be noted that while SFA in the diet have been classified as hypercholesterolemic, the various fatty acids in this classification vary in chain length and all SFA do not have the same effects on plasma lipoprotein levels. For that reason it is necessary to consider the effects of the major dietary SFA separately in order to define their respective effects.

A. LAURIC ACID (C12:0)

Lauric acid is a major fatty acid found in "tropical oils," i.e., palm kernel oil and coconut oil, and appears to have a substantial hypercholesterolemic effect in humans and animals; however, there are only limited data available on this question and they have not always been consistent. One of the reasons for the uncertainties regarding the effects of lauric acid intake on plasma lipoprotein levels is that there are only a few studies reporting the results of comparisons of different SFA; most studies have compared a SFA, such as coconut oil, to a PUFA, but not to other SFA, which vary in fatty acid chain length.

Studies of the effect of lauric acid intake on plasma cholesterol levels have resulted in conflicting reports of it being as hypercholesterolemic as palmitic acid (Keys *et al.*, 1965) and as having only a small effect on plasma cholesterol (Hegsted *et al.*, 1965). Studies in animal models (rats, hamsters, guinea pigs, rabbits, and nonhuman primates) have shown that intake of coconut oil (48% lauric acid) has a pronounced hypercholesterolemic effect when compared to PUFA. Data from studies in guinea pigs fed diets containing 35% (energy percent; ene) palm kernel oil (49% lauric acid) indicate a hypercholesterolemic response as compared to lard or beef tallow (M. L. Fernandez, E. C. K. Lin, and D. J. McNamara, unpublished observations). What is unclear from the data is whether the hypercholesterolemic effect is due to the high lauric acid content of these fats or to the myristic acid content.

Studies by Hayes and colleagues in nonhuman primates (Hayes *et al.*, 1991) and in hamsters (Lindsey *et al.*, 1990) compared diets of similar fatty acid composition with the primary variable being a shift from lauric acid to palmitic acid while maintaining a constant P:S ratio. In both studies there was a reduction in plasma total and LDL cholesterol levels upon changing to the palmitic acid-containing diet. In the hamster, the shift from a lauric acid-rich to a palmitic acid-rich diet lowered the LDL:HDL ratio and increased hepatic apoA-I and LDL receptor mRNA levels (Lindsey *et al.*, 1990). The data suggest that within the class of SFA, lauric acid has a hypercholesterolemic effect in animals, and probably in man; however, more detailed studies investigating specific comparisons of different chain lengths of SFA as compared to lauric acid are needed to resolve the uncertainties regarding metabolic effects of these SFA (Grundy and Denke, 1990).

B. MYRISTIC ACID (C14:0)

Most diets contain relatively small amounts of myristic acid (Fig. 2), with the major source of dietary myristic acid from butter fat. While metabolic ward studies indicated that myristic acid intake increased plasma cholesterol levels (Keys *et al.*, 1965; Hegsted *et al.*, 1965), the degree of response compared to palmitic acid was unclear. There have been no studies specifically addressing the relative plasma cholesterol response to myristic acid intake relative to either lauric or palmitic acids in animal models or humans. Considering the relatively low intake of myristic acid in the American diet, its potential contribution to elevated plasma cholesterol levels is probably small.

C. PALMITIC ACID (C16:0)

Palmitic acid is the major SFA in most diets (Fig. 2), and metabolic studies by Keys *et al.* (1965) and Hegsted *et al.* (1965) clearly demonstrated a hypercholesterolemic response when palmitic acid replaced either PUFA or carbohydrates in the diet. Studies in humans have reported similar findings for plasma total and LDL cholesterol levels (Mattson and Grundy, 1985; Grundy and Vega, 1988; Bonanome and Grundy, 1988). While some animal studies suggest that intake of palmitic acid may not be as hypercholesterolemic as lauric and myristic acids (Hayes *et al.*, 1991; Lindsey *et al.*, 1990), studies consistently demonstrate that intake of palmitic acid, as compared to MUFA or PUFA, or to carbohydrate, increase plasma total and LDL cholesterol levels. Whether palmitic acid intake is more or less hypercholesterolemic than intake of lauric or myristic acids is a fine point of scientific interest but has little relevance to the public health issues regarding dietary SFA and elevated plasma cholesterol levels, since palmitic acid is the major SFA in the diet. Dietary interventions to lower saturated fat intake will by necessity result in a decrease in palmitic acid consumption and, based on the available evidence, will result in some degree of plasma cholesterol lowering.

D. STEARIC ACID (C18:0)

While it is generally held that SFA in the diet increase plasma cholesterol levels, the early clinical studies of dietary fat effects on plasma cholesterol suggested that cocoa butter, rich in stearic acid, did not raise cholesterol levels to the same extent as lauric, myristic, or palmitic acids (Ahrens *et al.*, 1957; Hegsted *et al.*, 1965; Keys *et al.*, 1965). Interest in testing the effects of stearic acid intake on plasma lipids and lipoproteins was reestablished following the report of Reiser *et al.* (1985), which indicated that intake of beef fat, high in

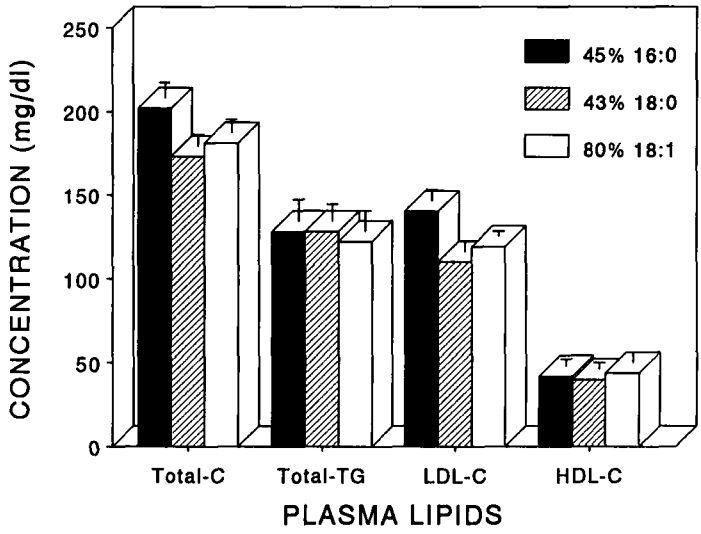

FIG. 4. Comparison of the effects of intake of palmitic, stearic, and oleic acids on plasma lipid and lipoprotein levels. Subjects (n = 11) were fed liquid formula diets containing 40% ene fat either high in palmitic acid (45% C16:0), stearic acid (43% C18:0), or oleic acid (80% C18:1) for 3 weeks after which plasma lipids and lipoproteins were determined. Plasma total cholesterol (C) and LDL cholesterol levels were significantly lower ($P < 0.02$) on both the stearic acid and oleic acid diets compared to the palmitic acid phase; HDL cholesterol levels were not significantly different. (After Bonanome and Grundy, 1988.)

stearic and oleic acids, resulted in reduced levels of plasma LDL and HDL cholesterol compared to coconut oil, and almost equivalent levels to values obtained during intake of safflower oil. Results from a metabolic ward study by Bonanome and Grundy (1988) in patients fed 40% ene fat diets rich in either palmitic acid, stearic acid, or oleic acid demonstrated that plasma LDL and HDL cholesterol levels and the LDL:HDL ratio were similar during the high oleic and high stearic acid feeding periods and significantly increased during intake of the high-palmitic acid diet (Fig. 4). The results of the Bonanome and Grundy (1988) study clearly demonstrate that not all SFA have the same cholesterol-raising potential and that intake of stearic acid, which can be converted *in vivo* to oleic acid, is not a typical plasma cholesterol-raising SFA.

E. MEDIUM-CHAIN FATTY ACIDS (C10:0 and C8:0)

There is little information available on the effects of medium-chain triglycerides (MCTs), C8:0 and C10:0, on plasma lipid and lipoprotein levels and metabolism. Since MCTs are absorbed directly via the portal circulation, there

is no contribution to the production of chylomicrons, which is why MCTs are prescribed for patients with hyperchylomicronemia due to LPL or apoC-II deficiencies. Studies indicate that MCT intake does not increase plasma cholesterol levels in humans or animal models (Hashim *et al.*, 1960; Beveridge *et al.*, 1959; Grande, 1962). Metabolic studies in hamsters fed diets containing 20% (by weight) fat, either MCTs or hydrogenated coconut oil, have shown that the MCT diet lowers plasma LDL cholesterol levels and reduces hepatic cholesterol synthesis, but has little effect on apoB/E receptor-mediated LDL catabolism or LDL production rates (Woollett *et al.*, 1989). The observed responses probably relate to the reduced flux of chylomicron fatty acids through the liver and the fact that hepatic cholesterol synthesis is increased by dietary fat flux. In view of the lack of chylomicron transport of MCTs to the liver, intake of MCTs results in a response metabolically similar to very low-fat, high-carbohydrate diets and has little effect on postprandial lipoprotein metabolic parameters due to the unique transport and catabolism of medium-chain fatty acids.

V. PLASMA LIPID/LIPOPROTEIN RESPONSES TO *n*-6 POLYUNSATURATED FATTY ACIDS

The plasma cholesterol-lowering effects of *n*-6 PUFA in the diet are well established. The early reports of Ahrens *et al.* (1957), Hegsted *et al.* (1965), Kinsell *et al.* (1952), and Keys *et al.* (1957) clearly established that intake of SFA, compared to intake of carbohydrates, raised serum cholesterol levels whereas PUFA intake lowered cholesterol concentrations. A long and extensive history of investigations on dietary fat saturation effects on plasma cholesterol levels has not contradicted these original observations (Grundy and Denke, 1990; McNamara, 1987; Kris-Etherton *et al.*, 1988; Goldberg and Schonfeld, 1985; Goodnight *et al.*, 1982). Detailed metabolic ward studies of the effects of dietary fat saturation on plasma cholesterol levels by Keys *et al.* (1965) and Hegsted *et al.* (1965) resulted in the development of formulas to quantify dietary fat effects on plasma total cholesterol levels (Table IV). In these equations only the effects of dietary SFA and PUFA and cholesterol are considered; at the time, MUFA were considered to have a neutral effect, similar to carbohydrates, on plasma cholesterol levels. As discussed below, it is now clear that neither carbohydrates nor MUFA can be considered as having a "neutral" effect on plasma lipoprotein levels. From the Keys and Hegsted equations it can be seen that the plasma cholesterol response to SFA is approximately double the response to PUFA. These equations have been widely used in various studies and the use of a dietary fat P:S ratio (ratio of PUFA to SFA in the diet) to describe the relative plasma cholesterol-raising or -lowering effect of fats is routinely provided by most studies. More recent studies have recognized the importance of MUFA in

TABLE IV

EQUATIONS TO ESTIMATE CHANGES IN PLASMA TOTAL CHOLESTEROL LEVELS RESULTING FROM MODIFICATIONS IN DIETARY FAT AND CHOLESTEROL[a]

Keys equation:
$\Delta\text{Cholesterol (mg/dl)} = 1.35(2\Delta S - \Delta P) + 1.5\Delta Z$
Hegsted equation:
$\Delta\text{Cholesterol (mg/dl)} = 2.16\Delta S - 1.65\Delta P + 0.097\Delta C$

[a] ΔS and ΔP are the changes in percentage of calories from saturated (S) and polyunsaturated (P) fat. ΔZ is the difference between the square root of the initial and subsequent intake of cholesterol (mg/1000 kcal) and ΔC is the difference in cholesterol intake (mg/1000 kcal). After Keys *et al.* (1965) and Hegsted *et al.* (1965).

determining plasma cholesterol levels and provide a P:M:S ratio to describe the dietary fatty acids.

A. MECHANISMS OF THE HYPOCHOLESTEROLEMIC EFFECTS OF DIETARY *n*-6 POLYUNSATURATED FATTY ACIDS

Numerous mechanisms have been suggested for the hypocholesterolemic action of PUFA in the diet, including changes in endogenous cholesterol metabolism and/or lipoprotein metabolism. There has been extensive research carried out over the past 30 years investigating the effects of PUFA intake on various aspects of cholesterol metabolism (absorption, synthesis, catabolism to bile acids, fecal excretion, and tissue distribution) and on the metabolism of the various lipoproteins (synthesis, composition, and catabolism) in both humans and a variety of animal model systems. The majority of data indicate that chronic intake of PUFA-rich diet does not affect endogenous cholesterol metabolism but rather exerts its hypocholesterolemic effect by modifications of both the synthesis and catabolism of plasma lipoproteins (McNamara, 1987; Grundy and Denke, 1990). Studies in humans under metabolic ward conditions and as free-living outpatients indicate that intake of PUFA, compared to SFA, has no effect on cholesterol absorption (Grundy and Ahrens, 1970; McNamara *et al.*, 1987), endogenous cholesterol synthesis (Grundy and Ahrens, 1980; Shepherd *et al.*, 1980), or bile acid synthesis (Grundy and Ahrens, 1970; Shepherd *et al.*, 1980). Animal model studies have for the most part resulted in similar findings (Ibrahim and McNamara, 1988; Fernandez *et al.*, 1990; Spady and Dietschy, 1988; Jones

et al., 1990; Bochenek and Rodgers, 1978; Kellogg, 1974). The available data indicate that the plasma cholesterol-lowering response to intake of PUFA in the diet results primarily from alterations in the synthesis and catabolism of the plasma lipoproteins and that if any changes in cholesterol metabolism occur, they are minimal and probably of little biological significance. One unique observation from animal studies is that intake of PUFA does result in increased levels of hepatic and extrahepatic cholesterol, suggesting that one effect of PUFA intake is a redistribution of tissue cholesterol. This metabolic response appears to be due to increased tissue uptake of LDL cholesterol in response to PUFA intake (Ibrahim and McNamara, 1988; Fernandez and McNamara, 1989; Spady and Dietschy, 1988; Walsh-Hentges *et al.*, 1985a,b).

B. POLYUNSATURATED FATTY ACIDS AND LIPOPROTEIN METABOLISM

1. Polyunsaturated Fatty Acid (n-6) Effects on Chylomicron Metabolism

Data regarding the effects of dietary fat saturation on postprandial lipoprotein metabolism are limited due to technical difficulties involved in the isolation of the various subfractions of triacylglycerol-rich lipoproteins. Human and animal studies suggest that dietary fat quality does not alter chylomicron synthesis and secretion rates but does affect the catabolism of chylomicrons and of chylomicron remnants.

Renner *et al.* (1986) reported that acute intestinal infusion of micelles containing either SFA or PUFA to rats resulted in lymph chylomicrons of similar size and composition. Studies by Davidson *et al.* (1987) of rats fed either butter fat or corn oil (30–35% by weight) indicate that dietary fat saturation has no effect on rates of apoA-I or apoB-48 synthesis by the intestine. These data are consistent with the report of Sorci-Thomas *et al.* (1989a,b) that intestinal apoB and apoA-I mRNA levels of African green monkeys were unaffected by a change in dietary fat quality from SFA to PUFA.

Analysis of the rate of chylomicron triacylglycerol synthesis in rats fed palm oil versus sunflower seed oil indicated no effect of fat saturation (Groot *et al.*, 1988); however, in these studies it was noted that chylomicrons from animals fed the PUFA sunflower seed oil were catabolized faster than chylomicrons from animals fed the SFA palm oil. Green *et al.* (1984) also found that chylomicron metabolism was faster in rats fed PUFA (corn oil) compared to SFA (palm oil). Similar data have been reported by Coiffier *et al.* (1987) demonstrating that chylomicrons from rats fed saturated mutton tallow were hydrolyzed more slowly *in vitro* compared to chylomicrons from rats fed polyunsaturated sunflower oil. In these studies (Coiffier *et al.*, 1987) it was found that intake of unsaturated fat was more effective than SFA in increasing LPL activity, whereas hepatic

triglyceride lipase (HTGL) was higher in SFA-fed animals compared to those on the unsaturated fat diet. The increased catabolism of chylomicrons in PUFA-fed animals appears to require chronic fat feeding in that analysis of the catabolism of chylomicrons from dogs given an acute feeding of corn oil or cream fat indicated a faster removal of cream-derived chylomicrons when tested in control dogs or rats (Nestel and Scow, 1964). Renner *et al.* (1986) reported that the clearance of palmitate-containing chylomicrons was slightly more rapid than that of chylomicrons containing linoleate in recipient rats. Similar findings have been reported by Green *et al.* (1984) indicating that the quality of fat fed to donor rats had less of an effect on chylomicron metabolism than did the metabolic state of the recipients, especially with regard to plasma triacylglycerol levels. These observations are consistent with the hypothesis that chylomicrons and plasma VLDL compete for the same sites of lipolytic activity and, in the presence of increased plasma VLDL triacylglycerol levels, the rate of chylomicron metabolism is decreased.

Human studies initially suggested that dietary fat saturation had little effect on postprandial plasma chylomicron concentrations and relative disappearance rates in normolipidemic individuals (Chait *et al.*, 1974; Aviram *et al.*, 1986). Analysis of the postprandial increments in plasma triacylglycerol levels above fasting levels indicated that observed differences in the postprandial levels between SFA and PUFA diet phases were due to differences in fasting levels rather than differences in alimentary lipemia (Chait *et al.*, 1974). More detailed studies investigating the time course of plasma apoB-48 levels (a marker for chylomicrons) in subjects fed 36% ene fat diets with P:S ratios of either 0.14 or 1.8 indicate an increased integrated plasma time course of apoB-48 due to intake of the SFA diet. The increased integrated plasma time course of apoB-48 appeared to be due to decreased clearance rather than increased secretion.

Studies by Weintraub *et al.* (1988) carried out in eight normolipidemic men demonstrated that feeding a diet composed of 42% ene P:S 1.39 fat compared to 42% ene P:S 0.07 fat decreased the postprandial chylomicron levels by 67% and that this response related more to the chronic intake of PUFA than to the fatty acid content of the acute fat challenge. These investigators reported that chylomicrons differed in their susceptibility to LPL hydrolysis *in vitro*, with PUFA-containing chylomicrons hydrolyzed faster than SFA-rich particles. The authors suggested that the effects of fat saturation on chylomicron metabolism differ between acute effects, which relate to susceptibility of chylomicrons to lipolysis, and chronic effects, which are directed at the catabolic system (Weintraub *et al.*, 1988).

From the available human and animal data it would seem that the major effects of dietary fat quality on the metabolism of chylomicrons and chylomicron remnants is an increased rate of catabolism of the particles during intake of a PUFA diet, whereas dietary fat quality apparently has no effect on intestinal apoB-48

or apoA-I synthesis or on chylomicron secretion. The increased catabolism of chylomicrons and their remnants during intake of PUFA in part can be explained by an increase in lipoprotein lipase (LPL) activity, resulting in a more rapid conversion of chylomicrons to remnants and their uptake via hepatic receptors. There is also evidence suggesting that unsaturated glycerides are more rapidly degraded by LPL and HTGL than saturated glycerides (Miller *et al.*, 1981; Weintraub *et al.*, 1988), which could play a role in chylomicron catabolism and hepatic uptake depending upon the fatty acid composition of the triacylglycerols derived from the dietary fat.

There is an obvious need to expand our understanding of the effects of dietary fat quality on the synthesis, secretion, composition, and catabolism of chylomicrons, especially in humans and as related to specific types of hyperlipoproteinemia. As discussed in more detail below, there is also a need to better define the effects of dietary fat saturation on the activities of enzymes involved in chylomicron catabolism, LPL and HTGL, and how differences in the size and composition of chylomicrons (Quarfordt and Goodman, 1966) affect the clearance of chylomicrons and chylomicron remnants from the plasma compartment.

2. *Polyunsaturated Fatty Acid (n-6) Effects on VLDL Metabolism*

a. Animal Studies. The effects of dietary PUFA versus SFA with and without added dietary cholesterol, on VLDL metabolism have been studied in a variety of animal species. Studies in rats generally indicate that dietary fat quality has little effect on VLDL triacylglycerol or cholesterol production rates. Kris-Etherton *et al.* (1984) reported no significant differences in the secretion of VLDL cholesterol by perfused livers of rats fed 10% ene safflower oil, corn oil, olive oil, or palm oil, although the data give a suggestion that VLDL cholesterol secretion rates of livers from rats fed the safflower oil diet were slightly increased, as reflected by a modest rise in cholesterol output and a significant increase in the VLDL cholesterol:protein ratio. Groot *et al.* (1988) reported that production rates of VLDL in rats fed a palm oil-containing diet (30% ene) were not different from values found in animals fed sunflower seed oil, and suggested that the increased plasma VLDL triacylglycerol levels in palm oil-fed rats were due to decreased catabolism. In contrast, Hostmark et al. (1980) observed that VLDL from liver perfusions of rats fed 10% (by weight) sunflower oil had a lower triacylglycerol:cholesterol ratio that VLDL from livers of rats fed 10% coconut oil; however, rates of liver output of VLDL protein, total cholesterol, and triacylglycerol were not significantly different between diet groups.

In striking contrast to the data from studies in rats, studies of VLDL secretion by isolated hepatocytes of hamsters fed diets containing 5% (by weight) of either palmitic acid or linoleic acid plus 0.1% cholesterol demonstrated that intake of

linoleic acid increased secretion of VLDL triacylglycerol and cholesterol compared to intake of palmitic acid (Ohtani *et al.*, 1990). Similar results have been obtained in gerbils fed either 31% ene coconut oil or safflower oil, wherein VLDL triacylglycerol, but not cholesterol, secretion was increased by intake of PUFA-containing diet (Nicolosi *et al.*, 1976).

Increased production of VLDL in animals fed diets containing PUFA as compared to SFA has also been reported from studies in various nonhuman primates: squirrel and cebus monkeys (Nicolosi *et al.*, 1977), African green monkeys (Johnson *et al.*, 1985), and rhesus monkeys (Khosla and Hayes, 1991). It should be noted, however, that interpretation of the data in large part depends on what components of the VLDL particles—apoproteins, cholesterol, or triacylglycerol—are being determined. Nicolosi *et al.* (1977) reported that VLDL triaclyglycerol secretion was increased in both squirrel and cebus monkeys fed either corn oil or safflower oil compared to animals fed coconut oil. Liver perfusion studies in African green monkeys fed 40% ene as either butter fat or safflower oil (plus 0.78 mg cholesterol/kcal) demonstrated that the rate of VLDL cholesterol secretion was increased in animals fed the safflower oil diet and that the secreted VLDL particles were smaller and contained more cholesteryl ester; rates of VLDL protein accumulation were not different between dietary fat groups (Johnson *et al.*, 1985). These data are consistent with the report of similar hepatic apoB mRNA levels and hepatic apoB secretion rates in African green monkeys fed the same test diets (Sorci-Thomas *et al.*, 1989a).

The finding of increased VLDL production in nonhuman primates fed diets containing PUFA presents a unique metabolic quandary: Since VLDL is the precursor of LDL, how do PUFA lower plasma LDL levels when the production of the precursor is increased? In part, the explanation could involve an increased rate of VLDL direct removal (i.e., removed from the plasma compartment without conversion to LDL). This is supported by studies of Khosla and Hayes (1991), who measured VLDL and LDL kinetics in rhesus monkeys fed 31% ene fat diets of coconut oil, P:S 0.17, or palm oil, P:S 0.35, to determine VLDL pool size, production, and fractional catabolic rates; absolute rates of VLDL direct removal and flux to LDL; and direct synthesis of LDL (i.e., direct LDL production by the liver independent of VLDL synthesis and catabolism). The results of their study indicate that feeding a palm oil-containing diet increased VLDL pool size, VLDL production rate, direct removal of VLDL from the plasma, and VLDL flux to LDL. The fractional catabolic rate of VLDL, the sum of direct removal and flux to LDL, was unaltered by dietary fat quality. The observed reduction in plasma LDL levels and pool size in monkeys on the palm oil-containing diet resulted from a decrease in the mass and proportion of LDL apoB derived from direct hepatic production independent of VLDL catabolism. The authors concluded from these studies that dietary saturated fat chain length has distinct effects on VLDL production rates, VLDL catabolism, and

VLDL-dependent and VLDL-independent production of LDL (Khosla and Hayes, 1991). Similar studies comparing SFA and PUFA would be very informative with regard to fat saturation effects on VLDL and LDL synthesis and on metabolic channeling of plasma VLDL.

b. Human Studies. There are very few studies of dietary fat saturation effects on VLDL metabolism in humans due to complexities of analysis of postprandial triacylglycerol-rich lipoproteins and fractionation of chylomicrons and VLDL and their remnant particles. Cortese *et al.* (1983) reported that intake of a 40% ene fat diet of P:S 3.8, compared to dietary fat of P:S 0.12, decreased the plasma VLDL apoB pool size and production rate with no change in the fractional catabolic rate. These authors concluded that the primary effect of dietary PUFA on VLDL metabolism was a decrease in VLDL apoB production rates. This conclusion is supported in part by the findings of Demacker *et al.* (1991) of a decreased integrated 24-hr concentration of apoB-100 in the $d <$ 1.019 fraction of subjects consuming a 36% ene fat diet of P:S 1.8 comparing to P:S 0.14, consistent with VLDL apoB-100 concentrations being lower throughout the day during intake of a PUFA-containing diet.

Demacker *et al.* (1991) present an interesting hypothesis to account for the interactions between chylomicrons, chylomicron remnants, VLDL, and VLDL remnants during intake of diets containing SFA by proposing that the delayed catabolism and clearance of chylomicrons and their remnants result in competition for the catabolism by LPL and clearance by hepatic receptors of VLDL and VLDL remnants, resulting in an increased rate of direct conversion of VLDL to LDL. Such a hypothesis is not, however, consistent with the findings of Khosla and Hayes (1991) of increased direct conversion of VLDL to LDL in rhesus monkeys fed a palm oil-containing diet as compared to one containing coconut oil. Whether this is a species difference or possibly related to the use of palm oil relative to coconut oil in the nonhuman primate studies is unknown. What does appear obvious is that our present state of knowledge regarding the effects of *n*-6 PUFA on VLDL production, intravascular processing, and catabolism, either via direct removal from the circulation or flux to LDL, especially in humans, is very limited.

There is convincing evidence that intake of PUFA-containing diets increases receptor-mediated catabolism of LDL (see below), yet dietary fat quality apparently has no effect on the fractional catabolic rate of VLDL (Cortese *et al.*, 1983). This could be due to differential effects of dietary fat saturation on the proportion of VLDL removed directly from the plasma compartment relative to the rate of conversion to LDL. It is also possible that dietary fat effects on the composition of newly secreted VLDL (Johnson *et al.*, 1985) alter intravascular processing, which, when coupled with changes in the activities of enzymes and proteins involved in intravascular processing and catabolism (LPL, HTGL, CETP,

and LDL receptor), affects the catabolic routes plasma VLDL particles take. Since dietary fat saturation alters plasma VLDL concentrations and composition, with the triacylglycerol:cholesterol ratio decreased during intake of unsaturated fat diets (Chait *et al.*, 1974), it is possible that the metabolic channeling of VLDL via intravascular processing to LDL versus hepatic catabolism could be strikingly different depending upon dietary fat saturation. Obviously, many unanswered questions remain to be investigated regarding the effects of dietary fatty acid saturation on VLDL metabolism.

3. *Polyunsaturated Fatty Acid (n-6) Effects on LDL Metabolism*

a. Animal Studies. It has been well documented in a wide variety of animal model systems that intake of *n*-6 PUFA lowers plasma total and LDL cholesterol levels compared to diets containing SFA. The majority of these studies have been carried out in various rodent models and in nonhuman primates and usually involve variations in the type and amount of dietary fat and in the amount of dietary cholesterol. Many of these reports are confounded by the use of inappropriate diet designs involving variable caloric densities and use of extremely high, pharmacological levels of dietary cholesterol, which in many cases probably overwhelms any specific effects of dietary fat saturation on plasma lipoprotein levels and metabolism. These confounding variables make interpretation of the data difficult, with the result that there are many conflicting conclusions in the literature and much confusion as to specific effects of fatty acid saturation.

The development of steady-state techniques for analysis of total and receptor-mediated LDL metabolism in animal models by Dietschy and colleagues (Spady *et al.*, 1985, 1986) has resulted in a number of reports on the effects of the type and amount of dietary fat on LDL metabolism in the hamster and the rat. In 1985, Spady and Dietschy (1985) reported that intake by hamsters of a commercial diet with 20% (by weight) safflower oil and 0.12% cholesterol resulted in lower plasma LDL cholesterol levels compared to a diet containing 20% hydrogenated coconut oil, and that the observed reduction in LDL was due to increased hepatic apoB/E (LDL) receptor-mediated clearance of plasma LDL. Subsequent studies in hamsters fed a commercial diet with either 20% (by weight) safflower oil or hydrogenated coconut oil, without added cholesterol, indicated that PUFA lowered plasma LDL concentrations and increased receptor-mediated LDL clearance (Spady and Dietschy, 1988).

The data derived from studies in hamsters consistently indicate an increased receptor-mediated hepatic catabolism of LDL in PUFA-fed animals, but studies in rats have indicated little if any effect of substituting 20% (by weight) safflower oil for 20% hydrogenated coconut oil on receptor-mediated catabolism of plasma LDL, even though plasma LDL levels are reduced (Ventura *et al.*, 1989; Spady and Woollett, 1990). The observed differences in responses of hamsters and rats

probably relate to the lipoprotein profiles exhibited by these two rodent species—rats have only 10–15% of the plasma total cholesterol as LDL cholesterol, whereas hamsters have 30–35% and thus may be more responsive to dietary manipulations involving changes in LDL synthesis and catabolism. Studies using hamster and rat models have provided clear evidence that the majority of plasma LDL clearance occurs via hepatic LDL receptor-mediated processes and that the plasma LDL-lowering effect of an increase in dietary PUFA at least in part relates to changes in LDL receptor-mediated hepatic catabolism of LDL.

Studies of the guinea pig, an animal with 50–70% of its plasma cholesterol as LDL cholesterol, have shown that intake of PUFA corn oil (35% ene), compared to SFA lard, significantly decreased plasma LDL cholesterol levels, increased LDL fractional catabolic rates, and decreased LDL apoB production rates (Fernandez *et al.*, 1992). The dietary fat effects on LDL catabolism were attributable to specific effects on LDL receptor-mediated LDL catabolism, since receptor-independent LDL uptake was found to be unaffected by dietary fat saturation. These studies also demonstrated a positive relationship between dietary fat saturation effects on hepatic LDL receptor number (B_{max}), measured *in vitro*, and receptor-mediated catabolism of LDL, measured in vivo (Fernandez *et al.*, 1992). Of interest was the finding that the source of tracer LDL (i.e., whether from an animal fed SFA lard or PUFA corn oil) had a significant effect on *in vivo* catabolism, with the PUFA-rich LDL from corn oil-fed animals having a higher fractional catabolic rate relative to LDL from lard-fed guinea pigs (Fernandez *et al.*, 1992). Effects of LDL compositional changes on metabolism will be discussed in detail later.

Studies by Portman *et al.* (1976) of squirrel monkeys fed diets containing 20% (by weight) fat, either butter oil or safflower oil, demonstrated that intake of the PUFA-rich diet increased the fractional catabolic rate of plasma LDL apoB and decreased LDL apoB production. These investigators reported that dietary fat quality had no effect on LDL composition and that the dietary group used as the source of LDL for labeling and kinetic measurements did not influence the fractional turnover rates of LDL (Portman *et al.*, 1976).

The effects of dietary fat saturation on LDL receptor-mediated and receptor-independent LDL catabolism in cebus monkeys fed 31% ene fat, either SFA coconut oil or PUFA corn oil, have been reported by Nicolosi *et al.* (1990). Intake of PUFA significantly decreased plasma total, VLDL + LDL, and HDL cholesterol levels and reduced plasma concentrations of apoB and apoA-I. The changes in plasma LDL and apoB levels were related to a PUFA-mediated increase in both receptor-mediated and receptor-independent LDL catabolism as compared to dietary SFA; LDL apoB production rates were similar for both groups of animals.

The majority of data from animal model studies indicate that shifting dietary fat quality from SFA to PUFA increases the fractional catabolic rate of plasma

LDL and, in a number of studies, decreases LDL apoB production rates. The increased fractional catabolic rate in PUFA-fed animals results from an increase in LDL receptor-mediated hepatic catabolism of LDL, with no evidence for an increased rate of catabolism by extrahepatic tissues. The increased LDL fractional catabolic rate is correlated with an increased number of hepatic LDL receptors in animals fed a PUFA-rich diet (Fernandez and McNamara, 1989, 1991a; Fernandez *et al.*, 1992). This increase in hepatic catabolism of LDL occurs in the presence of a PUFA-mediated increase in hepatic cholesteryl ester content and in most cases an identical rate of hepatic cholesterol synthesis (Spady and Dietschy, 1985; Fernandez *et al.*, 1990, 1992; Fernandez and McNamara, 1991a).

b. Human Studies. The effects of SFA versus PUFA on LDL metabolism have been the subject of some debate due to somewhat conflicting reports in the literature. Shepherd *et al.* (1980) studied eight male subjects fed 40% ene fat diets with P:S ratios of either 0.25 or 4.0 (400 mg/day dietary cholesterol) and reported that the high-*n*-6 PUFA diet increased the LDL fractional catabolic rate but had no effect on LDL apoB production rates. In contrast, Turner *et al.* (1981) reported LDL kinetic studies in normolipidemic and hypercholesterolemic subjects fed 40% ene fat diets with P:S ratios of either 0.2 or 8.0 (150 mg/day dietary cholesterol) and found that intake of the *n*-6 PUFA diet decreased LDL apoB production in the normolipidemic subjects but had no effect on LDL fractional catabolic rates. These same authors reported that in hypercholesterolemic subjects the high-PUFA diet significantly increased LDL catabolism and decreased LDL production rates (Turner *et al.*, 1981). Studies by Cortese *et al.* (1983) in normolipidemic subjects found that a shift from SFA to PUFA (45% ene, P:S 0.12 versus 3.8, 365 mg/day dietary cholesterol) resulted in a significant reduction in LDL apoB production but not catabolism. A total of 26 LDL kinetic measurements were carried out in these three studies, and when the data were combined and analyzed it could be concluded that exchange of PUFA for SFA in the diet results in a significant increase in LDL fractional catabolic rate (0.306 ± 0.067 versus 0.335 ± 0.073 day^{-1}, $P < 0.003$) and a significant decrease in LDL apoB production rate (13.13 ± 2.49 versus 11.45 ± 1.94 mg/kg/day, $P < 0.0001$).

These data are consistent with a number of reports on the effects of dietary fat saturation on LDL kinetics in animal models (Portman *et al.*, 1976; Fernandez *et al.*, 1991; Nicolosi *et al.*, 1990; Spady and Dietschy, 1988), which indicate that intake of PUFA in the diet results in increased LDL catabolism and decreased LDL apoB production. A currently unresolved question is whether the decreased LDL apoB production due to intake of PUFA is due to a decreased production of VLDL apoB, decreased conversion of VLDL apoB to LDL, increased receptor-mediated direct removal of VLDL and IDL, decreased direct secretion of LDL, or a combination of these alterations in the metabolism of apoB-containing

lipoproteins (Grundy and Denke, 1990). There are limited data suggesting that intake of *n*-6 PUFA decreases VLDL apoB production rates without affecting the fractional catabolic rate (Cortese *et al.*, 1983); unfortunately, the data are from only four subjects and, as noted above, the investigators did not observe an effect of PUFA intake on LDL apoB turnover. Therefore, it remains uncertain whether dietary intake of *n*-6 PUFA increases the fractional catabolic rates of VLDL and IDL apoB in man (Grundy and Denke, 1990), and further investigations on this question are required.

An additional complication in these kinetic studies is that recent data indicate that alterations in the chemical composition of plasma LDL mediated by dietary fat saturation result in particles that have different *in vivo* and *in vitro* metabolic characteristics. In other words, fat saturation not only affects the liver's ability to synthesize and metabolize LDL, but also affects the intravascular processing and interaction of LDL with hepatic LDL receptors. These changes affect rates of LDL catabolism *in vivo* as discussed below.

c. Regulation of LDL (apoB/E) Receptor Mass and Gene Expression. There is considerable uncertainty at present regarding the effects of dietary fat saturation on expression of the LDL receptor gene, and two hypotheses have been proposed to explain the increased LDL fractional catabolic rate in humans and animals fed PUFA. Studies by Fox *et al.* (1987) of baboons indicated that intake of SFA (25% ene coconut oil plus 1% cholesterol), compared to PUFA (25% ene peanut oil or olive oil plus 1% cholesterol), resulted in significantly lower levels of hepatic LDL receptor mRNA. These data suggest that one mechanism involved in the hypercholesterolemic response to SFA in the diet is suppression of LDL receptor gene expression, resulting in reduced synthesis of receptors —a concept consistent with the observation of a reduction in receptor-mediated LDL binding to hepatic membranes *in vitro* (Ibrahim and McNamara, 1988; Fernandez and McNamara, 1989, 1991a; Fernandez *et al.*, 1992), decreased receptor-mediated hepatic LDL clearance *in vivo* (Spady and Dietschy, 1988), and decreased receptor-mediated LDL fractional catabolic rates *in vivo* (Nicolosi *et al.*, 1990; Fernandez *et al.*, 1992 in animals fed SFA.

In contrast to the findings of Fox *et al* (1987), Sorci-Thomas *et al.* (1989a) reported that, in African green monkeys fed SFA (40% ene, P:S 0.3) or PUFA (40% ene, P:S 2.2), hepatic LDL receptor mRNA exhibited similar abundance. Testing the same diets with added cholesterol (0.8 mg/kcal) resulted in reduced LDL receptor mRNA levels independent of the type of dietary fat (Sorci-Thomas *et al.*, 1989a). These authors concluded that differences in plasma LDL cholesterol levels between animals fed SFA and PUFA could not be explained by changes in LDL receptor mRNA abundance and that alternative theories, such as alterations in membrane fluidity or changes in translational or posttranslational events, were required to explain the increased LDL catabolism resulting from

intake of PUFA (Sorci-Thomas *et al.*, 1989a). Similar data have been reported by Lindsey *et al.* (1990), who found that hamsters fed 13% ene fat diets of varying fatty acid chain lengths and degrees of saturation had similar levels of hepatic LDL receptor mRNA for dietary fat mixes of P:S 0.38 and 1.04; however, intake of a diet rich in C12:0 and C14:0 and low in MUFA resulted in lower levels of receptor mRNA. In none of these studies were the masses of hepatic LDL receptors measured and therefore the mRNA data must be viewed as preliminary until rates of receptor synthesis and receptor mass values are available to determine whether dietary fat saturation is altering transcription, translation, posttranslational events, or receptor function, possibly by changes in rates of receptor binding, internalization, recycling, or clustering in coated pits.

The concept of dietary fat saturation altering membrane fluidity, which in turn could affect the efficiency of membrane recycling or clustering in coated pits, is supported by a variety of experimental data. Gavigan and Knight (1981) reported that when the degree of unsaturation of phospholipid fatty acids of fibroblasts was increased, there was an associated increase in LDL degradation, both LDL receptor mediated and receptor independent, without a significant increase in the number of cell surface LDL receptors. Similar data have been reported by Loscalzo *et al.* (1987), suggesting that *in vitro* enrichment of human mononuclear cell membrane phospholipids with unsaturated fatty acids, either oleate or linoleate, increased membrane fluidity and LDL receptor-mediated LDL internalization and degradation. In these same studies it was found that membrane enrichment with stearate had no effect on either membrane fluidity or receptor-specific LDL metabolism (Loscalzo *et al.*, 1987).

Analysis of rates of LDL degradation in isolated mononuclear cells of cebus monkeys fed either coconut oil or corn oil indicated an increased LDL degradation rate in cells from corn oil-fed animals. Of interest was the observation that the rate of LDL degradation by isolated mononuclear cells was related to changes in the ratio of membrane unsaturated to saturated fatty acids and fluidity (Kuo *et al.*, 1989). Subsequent studies by Kuo *et al.* (1990a,b) found that *in vitro* alterations in membrane fatty acyl composition of U937 monocytes and HepG2 hepatocytes resulted in significant changes in LDL metabolism. Enrichment of cell membranes with cis-unsaturated fatty acids augmented LDL binding, internalization, and degradation compared to enrichment with saturated fatty acids. Enrichment of membranes with linoleate, compared to stearate, increased both LDL receptor affinity for LDL (K_d) and apparent receptor number (B_{max}). Studies in HepG2 hepatocytes, comparing enrichment with stearate or palmitate, indicated that receptor affinity and number were reduced when membranes were enriched with the shorter chain C16:0 fatty acid (Kuo *et al.*, 1990a). In all cases, increases in membrane fluidity were related to enhanced LDL receptor function; the greater the fluidity, the greater the receptor-mediated binding, internalization, and degradation of LDL.

The results of alterations in membrane fatty acid composition achieved by *in vitro* modifications differ from results of studies using isolated membranes from animals fed different dietary fatty acids. *In vitro* studies of LDL binding to hepatic membranes of guinea pigs fed SFA (lard), MUFA (olive oil), and PUFA (corn oil) indicate similar receptor affinities (K_d) for LDL by all membrane preparations and increased receptor number (B_{max}) for hepatic membranes from animals fed a 35% ene corn oil-based semipurified diet (Fernandez and McNamara, 1991b). While these data do not indicate changes in membrane receptor affinity for LDL due to changes in membrane fatty acids, this does not exclude the possibility that increased membrane fluidity results in an increased rate of receptor recycling, which would account for a higher number of LDL receptors on the cell surface at any one time. *In vivo* LDL turnover studies in guinea pigs fed the SFA, MUFA, and PUFA fats indicated a significant ($r = 0.994$) correlation between LDL receptor number, measured *in vitro,* and receptor-mediated LDL catabolism, measured *in vivo,* which could be explained by an increased number of receptors or an increased rate of receptor recycling (Fernandez *et al.*, 1992).

Whether PUFA intake increases LDL catabolism by increasing LDL receptor number or receptor function cannot be answered at present. There is evidence indicating that increased membrane fluidity does affect receptor function; whether this is the primary mechanism for the hypocholesterolemic effect of PUFA or also involves a total increase in the number of cellular LDL receptors must still be determined. There is little doubt that PUFA in the diet lower plasma LDL levels by increasing receptor-mediated LDL catabolism, coupled with a decrease in LDL apoB synthesis, whether directly by decreasing VLDL and/or LDL apoB synthesis or indirectly by increasing VLDL and IDL hepatic catabolism, with a decreased conversion to LDL.

d. Polyunsaturated Dietary Fatty Acid Effects on LDL Composition and Size. There is one additional factor involved in the plasma LDL-lowering response to intake of *n*-6 PUFA: dietary fat-mediated alterations in the size and composition of LDL appear to have significant effects on LDL metabolism. Both human and animal studies indicate that intake of PUFA results in smaller, cholesteryl ester-poor LDL particles that exhibit different metabolic characteristics. The original concept of dietary PUFA modifying LDL composition was proposed by Spritz and Mishkel (1969) and postulates that lipids enriched with PUFA occupy more space than those containing SFA and as such the LDL particles contain fewer cholesteryl esters. A number of studies have presented data consistent with this hypothesis (Shepherd *et al.*, 1980; Durrington *et al.*, 1977; Pownall *et al.*, 1980; Vessby *et al.*, 1980), while others have been unable to verify it (Vega *et al.*, 1982; Kuksis *et al.*, 1982). Consideration of all the data suggests that intake of PUFA not only reduces LDL apoB levels, but also results

in some degree of cholesteryl ester depletion of the LDL particle as compared to dietary intake of saturated fatty acids. Studies in various animal model systems have reported similar findings (Ibrahim and McNamara, 1988; Fernandez and McNamara, 1989, 1991a; Nicolosi *et al.*, 1990).

Data from both *in vitro* cell culture studies and *in vitro* metabolic studies indicate that dietary fat-mediated changes in LDL composition result in significant changes in the interactions between LDL and their receptors. Studies by Baudet *et al.* (1984) presented evidence indicating that dietary fat saturation altered the composition and *in vitro* cellular metabolism of LDL, with SFA intake resulting in LDL particles having decreased rates of binding, internalization, and degradation by human skin fibroblasts compared to LDL obtained during intake of PUFA. The data also indicated that alterations in LDL composition affected the normal pattern of *in vitro* cellular regulatory responses—sterol synthesis and intracellular cholesterol esterification (Baudet *et al.*, 1984).

In an analysis of the effects of dietary fat saturation on *in vitro* LDL degradation of mononuclear cells of cebus monkeys, animals were fed 31% ene fat diets, either coconut oil or corn oil, and the isolated LDL and mononuclear cells were studied in a cross-over design. While dietary fat saturation had a significant effect on rates of LDL degradation by the mononuclear cells, the source of LDL was also a significant determinant. As compared to LDL from animals fed SFA coconut oil, LDL from corn oil-fed cebus monkeys were degraded more rapidly, irrespective of the source of the mononuclear cells tested. Dietary fat saturation did not affect the affinity of the ligand for the receptor; however, LDL isolated from coconut oil-fed animals exhibited a decreased maximal binding to the mononuclear cells (Kuo *et al.*, 1989).

Studies in guinea pigs fed 19% ene or 35% ene corn oil, olive oil, or lard demonstrated that intake of the PUFA diet results in plasma LDL particles that have a lower percentage of cholesteryl ester, a higher density, and a reduced diameter compared to LDL isolated from animals fed either MUFA or SFA. *In vitro* binding studies of LDL isolated from each of these dietary fat groups using a standardized membrane preparation failed to support the theory that changes in LDL composition induced by dietary fat saturation affect the affinity or binding of LDL to the LDL receptors (Fernandez and McNamara, 1989, 1991a). However, subsequent studies of *in vivo* LDL kinetics in guinea pigs fed the same dietary fats (35% ene) did demonstrate a significant effect of LDL composition on LDL fractional catabolic rates when measured in guinea pigs fed a nonpurified commercial diet (Fernandez *et al.*, 1992). LDL particles from animals fed the lard- and olive oil-based diets had similar compositions and fractional catabolic rates. In contrast, LDL from guinea pigs fed the corn oil-based diet, which had a reduced cholesteryl ester content and a higher density, exhibited a significantly greater fractional turnover rate in animals on the commercial diet. These data are consistent with the smaller, more dense LDL particle having an increased

rate of catabolism *in vivo*, which can add to the efficacy of PUFA in lowering plasma LDL levels.

These data provide consistent evidence that alterations in LDL composition due to dietary fat saturation can significantly alter LDL metabolism *in vitro* and *in vivo*. There are a number of possible explanations why dietary fat saturation alters the cholesteryl ester-to-protein ratio of plasma LDL. One possibility is that the reduced plasma residence time of the particle, due to increased LDL receptor-mediated catabolism, results in a net decrease in CETP-catalyzed transfer of HDL cholesteryl ester to LDL. It is also possible that dietary fat saturation alters plasma CETP activity, as has been shown for high-fat, high-cholesterol diets (Stein *et al.*, 1990). The combination of these two factors would be expected to alter LDL intravascular processing and composition.

Studies by Kinoshita *et al.* (1990) have demonstrated that incubation of VLDL and LDL in the presence of plasma lipid transfer proteins results in LDL particles with a smaller diameter, a decreased cholesteryl ester content, an increased percentage of triacylglycerol, and a decreased affinity for the LDL receptor. These authors concluded that altering the core lipids of LDL results in changes in LDL size and in the conformation of several regions of apoB, and that these changes in conformation can modulate the interactions of apoB with the LDL receptor (Kinoshita *et al.*, 1990). Similar data have been reported by Chait *et al.* (1984) demonstrating that LDL particles enriched in triacylglycerol and depleted in cholesteryl ester, following incubation with lipid transfer proteins, exhibit decreased uptake and degradation by fibroblasts and macrophages and decreased regulatory effects on LDL receptor expression. Modifications of the intravascular processing of LDL induced by the intake of PUFA, resulting in smaller, cholesteryl ester-poor particles, would in part explain some of the dietary fat effects on LDL composition and metabolism reported in the literature (Kuo *et al.*, 1989; Fernandez *et al.*, 1992; Baudet *et al.*, 1984). Obviously dietary fatty acids can alter *in vivo* LDL metabolism not only by affecting the expression of hepatic LDL receptors but also by affecting the composition, size, density, and fluidity of LDL, thereby altering the interaction of the ligand with its receptor. Further studies are needed to better define the role of LDL compositional changes in mediating the dietary fat effects on LDL levels and metabolism.

4. Polyunsaturated Fatty Acid (n-6) Effects on HDL Metabolism

There has been some disagreement regarding the effects of dietary PUFA on plasma HDL levels and rates of apoA-I synthesis and catabolism. Considering the significant role HDL levels have in determining CHD risk (Gordon *et al.*, 1989), it is essential that the consequences of modifying the fatty acid distribution of the diet are clearly documented regarding changes in atherogenic LDL levels and in the protective HDL levels. This question has led to some debate regarding

the relative values of PUFA versus MUFA diets and what constitutes an appropriate level of dietary fat intake (Grundy, 1989).

a. Animal Studies. Extrapolation of most of the studies of the effects of dietary fat saturation on HDL levels and metabolism in animal models to humans is confounded by the fact that most animal model systems—rats, hamsters, monkeys, and baboons—have HDL as the predominant plasma cholesterol-transporting lipoprotein, a condition distinctly different from that in humans, in which HDL carry an average of approximately 25% of the plasma cholesterol. An additional complicating factor is that plasma CETP activity varies from almost undetectable in some animals to very active in others (Ha and Barter, 1982), and such differences no doubt have a significant effect on HDL apoprotein and cholesterol metabolism.

Studies in nonhuman primates have demonstrated that PUFA-rich diets lower plasma HDL cholesterol and apoA-I levels compared to diets containing SFA. Studies by Rudel and co-workers (Parks and Rudel, 1982; Johnson *et al.*, 1986; Babiak *et al.*, 1988; Sorci-Thomas *et al.*, 1989b) in African green monkeys have shown that intake of safflower oil (40% ene, P:S 0.3) lowers plasma HDL cholesterol levels (6–10%) and apoA-I levels (1–6%) compared to lard intake (40% ene, P:S 2.2). Johnson *et al.* (1986) found in liver perfusion studies of PUFA-fed monkeys that hepatic secretion of HDL was reduced relative to SFA-fed animals. Subsequent studies indicated that PUFA intake, compared to SFA intake, lowers hepatic apoA-I secretion rates but does not affect hepatic or intestinal apoA-I or apoA-II mRNA levels (Sorci-Thomas *et al.*, 1989b). There is some evidence to suggest that intake of PUFA lowers intestinal secretion of chylomicron apoA-I (Parks and Rudel, 1982), as suggested by compositional analysis of lymph chylomicrons.

Studies of dietary fat saturation effects on HDL apoprotein kinetics in rhesus monkeys have been reported by Chong *et al.* (1987) and indicate that SFA in the diet (30% ene coconut oil) lower apoA-I and apoA-II fractional catabolic rates, compared to PUFA (30% ene corn oil). Dietary fat saturation had no effect on rates of apoA-I and apoA-II production. These data are consistent with the report of Parks and Rudel (1982) of increased catabolism of HDL and chylomicron apoA-I in PUFA-fed African green monkeys without alterations in apoA-I production; however, the data are strikingly different from the findings of liver perfusion studies, which suggest decreased secretion (Johnson *et al.*, 1986). It has been demonstrated that the metabolism of HDL apoproteins varies significantly among different species of nonhuman primates (Sorci-Thomas et al., 1988) and this in part could explain some of the variances in response to changes in dietary fat saturation; however, it cannot explain the observed differences within species. The fact that apoA-I is synthesized in the liver, intestine, and other organs no doubt complicates the kinetic analyses, and if diet alters the

free apoA-I pool, these combined effects could account for some of the discrepancies.

b. Human Studies. The effects of dietary fat saturation on plasma HDL cholesterol concentrations, the relative proportions of HDL_2 and HDL_3, and apoA-I levels have been an area of considerable investigation and debate because of the evidence that plasma HDL levels are important determinants of cardiovascular disease risk. The evidence suggests that both the type and the amount of dietary fatty acids affect plasma HDL cholesterol levels, with plasma HDL decreasing with intake of diets high in PUFA and with intake of extremely low-fat diets; however, there remains considerable debate on the extent and significance of these observations (Table V).

In 1978, Shepherd *et al.* (1978) reported studies comparing the effects of two 40% ene fat diets, P:S 0.25 versus 4.0, on plasma HDL cholesterol levels and apoA-I metabolism in four male subjects. The data demonstrated that intake of the high-P:S diet lowered all the plasma lipoproteins, including plasma concentrations of HDL cholesterol (-32%) and apoA-I (-21%). Metabolic studies of HDL–apoA-I kinetics indicated that the high-P:S diet increased the fractional catabolic rate by 10% and reduced the production rate by 26%. These authors reported that the effect of changes in dietary fat saturation were specific for HDL_2 cholesterol and that there were no changes in the composition of total HDL. Since these studies were reported, there have been numerous studies on the effects of dietary fat quantity and quality on plasma HDL cholesterol and apoprotein levels but no studies on the effects of an isocaloric exchange of PUFA for SFA on HDL kinetics in humans. It is surprising that this single study in four patients represents the only data available addressing this very important question.

Studies by Fisher *et al.* (1983) and Zanni *et al.* (1987) have shown that intake of PUFA-rich diets (31% ene corn oil) results in significant reductions in plasma HDL cholesterol levels compared to diets high in SFA (31% ene coconut oil or lard). The data also indicate that the high-P:S diet lowers plasma apoA-I levels (Zanni *et al.*, 1987). Similar data were reported by Wolf and Grundy (1983) comparing the effects of 30% ene fat diets with P:M:S ratios of either 1:1:2 or 1:1:1 on plasma HDL cholesterol levels in five subjects. Intake of the P:S 1.0 diet resulted in significantly lower HDL levels than intake of the P:S 0.5 diet. In contrast, Vessby *et al.* (1980), Kraemer *et al.* (1982), Becker *et al.* (1983), and Blanton *et al.* (1984) reported that isocaloric fatty acid changes from low- to high-P:S diets had no significant effect on plasma HDL cholesterol levels. As shown in Table V, the majority of recent studies indicate that when there is an isocaloric exchange of PUFA for SFA, in the range of 30–40% ene and between P:S 0.2 and 2.0, plasma total HDL cholesterol levels are not significantly lowered (McNamara *et al.*, 1987; Mensink *et al.*, 1989; Wardlaw and Snook, 1990;

TABLE V

STUDIES OF DIETARY FAT SATURATION EFFECTS ON PLASMA HDL CHOLESTEROL AND APOLIPOPROTEINS

Fat (% ene)[a]	SFA	MUFA	PUFA	HDL-C (mg/dl)	DHL_2-C (mg/dl)	HDL_3-C (mg/dl)	ApoA-I (mg/dl)	Apo-A-II (mg/dl)	Reference
45	25	115	5	38 ± 4	—	—	88 ± 16	—	Vessby *et al.* (1980)
44	11	13	21	36 ± 3	—	—	83 ± 18	—	(n = 30)
29	8	10	11	52 ± 8	31 ± 10	24 ± 3	140 ± 25	—	Brussaard *et al.*
19	7	8	4	50 ± 8	26 ± 10	22 ± 3	139 ± 25	—	(1982) (n = 38)
37	22	12	3	55 ± 3	—	—	166 ± 5	46 ± 1	Ehnholm *et al.* (1982)
24	9	6	9	46 ± 2	—	—	157 ± 2	49 ± 1	(n = 54)
40	20	16	4	40 ± 11	—	—	101 ± 25	—	Becker *et al.* (1983)
40	4	32	4	43 ± 9	—	—	114 ± 31	—	(n = 12)
38	20	13	5	51 ± 2	32 ± 2	19 ± 1	142 ± 6	41 ± 1	Ehnholm *et al.* (1984)
19	7	6	6	46 ± 2	28 ± 2	19 ± 1	118 ± 3	37 ± 3	(n = 38)
35	18	12	5	51 ± 2	33 ± 2	19 ± 1	139 ± 3	39 ± 1	
37	20	13	4	56 ± 5	29 ± 3	27 ± 1	—	—	Kushi *et al.* (1985)
22	9	7	6	46 ± 3	21 ± 2	27 ± 1	—	—	(n = 38)
37	20	13	4	66 ± 3	43 ± 3	24 ± 1	—	—	Kushi *et al.* (1985)
22	9	7	6	55 ± 3	33 ± 2	22 ± 1	—	—	(n = 39)
40	20	12	8	—	18 ± 9	34 ± 6	108 ± 15	98	Mensink *et al.* (1989)
22	7	9	6	—	15	30		41 ± 3 41	(n = 24)

40	20	12	8	—	19 ± 9	34 ± 6	109 ± 13	44 ± 5	Mensink *et al.* (1989)
41	10	24	7	—	18	34	106	40	(n = 24)
40	21	14	5	43 ± 2	—	—	132 ± 6	—	Wardlaw and Snook
41	7	28	6	43 ± 2	—	—	120 ± 5	—	(1990) (n = 20)
41	8	14	19	43 ± 2	—	—	126 ± 4	—	
30	7	19	4	58 ± 2	19 ± 1	39 ± 1	—	—	Sola *et al.* (1990)
30	7	10	13	61 ± 1	23 ± 1	38 ± 1	—	—	(n = 12)
30	7	10	13	67 ± 1	23 ± 2	44 ± 1	—	—	
31	16	11	4	69 ± 4	26 ± 2	43 ± 1	—	—	
43	24	17	2	56 ± 13	—	—	139 ± 22	30 ± 7	Brinton *et al.* (1990)
8	2	4	2	40 ± 10	—	—	107 ± 22	30 ± 11	(n = 13)
34	7	19	8	45 ± 1	—	—	131 ± 30	—	Chan *et al.* (1991)
34	6	19	8	44 ± 1	—	—	130 ± 30	—	(n = 8)
34	7	9	18	45 ± 1	—	—	127 ± 30	—	
34	7	10	17	44 ± 1	—	—	124 ± 30	—	
33	14	14	5	45 ± 1	—	—	140 ± 20	—	
373	21		3	42 ± 9	17 ± 7	25 ± 5	117 ± 23	—	Fumeron *et al.*
39	12	14	13	40 ± 8	15 ± 6	25 ± 4	112 ± 17	—	(1991b) (n = 36)
42	17	19	6	37 ± 2	—	—	120 ± 30	—	Iacono and
24	10	10	4	36 ± 2	—	—	143 ± 50	—	Dougherty (1991)
29	9	9	11	37 ± 2	—	—	149 ± 70	—	(n = 11)

[a]ene, Energy percent.

Fumeron *et al.*, 1991b; Chan *et al.*, 1991). While total HDL cholesterol levels did not vary significantly in these studies, the plasma levels of apoA-I and of HDL_2 cholesterol were lowered in participants on the high-P:S diet in some of the studies (Vessby *et al.*, 1980; Sola *et al.*, 1990; Fumeron *et al.*, 1991a; Chan *et al.*, 1991).

As will be discussed in more detail below, most of the studies investigating dietary fat effects on plasma HDL levels vary both in the type and the amount of fat, and there is a limited data base of cross-over studies comparing the effects of isocaloric exchange of PUFA for SFA on plasma levels of total HDL, HDL subfractions, and apolipoproteins. The available data suggest that when dietary fat quality changes from a low to high P:S ratio, within ranges comparable to dietary changes the public might make, that there is a small decrease in plasma HDL_2 cholesterol and apoA-I concentrations, which, considering the limits of the analytical tests available and interindividual variability, is usually found to be not significant. As will be noted later, the amount of dietary fat appears to have a greater effect on plasma HDL levels than does the type of dietary fat.

c. HDL Receptor. Various studies have presented evidence for the existence of cell membrane HDL-binding proteins (Graham and Oram, 1987), and animal studies suggest that HDL binding to cell membranes is regulated by dietary fat saturation. Hepatic membranes isolated from guinea pigs fed 19% ene corn oil, olive oil, and lard-based diets demonstrate increasing HDL binding as the degree of dietary fat saturation decreases from SFA to MUFA to PUFA (Fernandez and McNamara, 1991b). Binding affinity values (K_d) are unaffected by fat saturation whereas the receptor number (B_{max}) increases from a low in lard-fed animals, intermediate in olive oil-fed guinea pigs, and a high during intake of corn oil. Whether the increased expression of HDL binding by hepatic membranes is due to increased numbers of receptors or, as suggested for LDL binding data, due to increased membrane fluidity, resulting in higher numbers being present at any one time on the membrane, remains unknown.

Similar observations have been reported from studies of HDL binding to rat adipocyte plasma membranes (Zsigmond *et al.*, 1990a,b). Intake of a 20% (by weight) sunflower oil diet, compared to a 20% (by weight) lard diet, increased the maximal binding capacity (B_{max}) of HDL_1 and HDL_2 to plasma membranes of both epididymal and perirenal adipocytes without altering the affinity constant (K_d) (Zsigmond *et al.*, 1990a). Dietary PUFA intake also significantly enhanced HDL_2 apoprotein and cholesteryl ester uptake by perirenal adipocytes as compared to intake of SFA (Zsigmond *et al.*, 1990b).

These data indicate that membrane binding of HDL is increased by intake of diets high in polyunsaturated fatty acids and in part could explain the observed increases in the fractional catabolic rate of apoA-I during intake of diets with high P:S ratios. As noted above for dietary fat effects on the LDL receptor, the

data are consistent with two potential mechanisms, either an increased expression of hepatic membrane HDL receptors or an increased membrane fluidity, resulting in a faster receptor recycling rate. The effects of PUFA-rich diets on the selective uptake of HDL cholesterol (Pittman *et al.*, 1987) and what role this might play in reverse cholesterol transport remain unknown.

5. *Polyunsaturated (n-6) Fatty Acid Effects on Intravascular Processing of Plasma Lipoproteins*

The effects of the amount and type of dietary fatty acids on the concentration of the plasma lipoproteins result not only from changes in the synthesis, composition, and catabolism of the particles but also from fat-mediated alterations of enzymes involved in the intravascular processing of the particles. Alterations in the activities of LPL and HTGL mediated by dietary fat quality and quantity can have significant effects on the catabolism and composition of the triacylglycerol-rich lipoproteins (chylomicrons, chylomicron remnants, and VLDL). In a similar manner, dietary fatty acids can influence the activities of plasma LCAT and CETP, which can affect plasma HDL cholesterol levels and the compositions of VLDL and LDL. In theory, changes in dietary fat quality and quantity can modify plasma lipoprotein levels by two complementary mechanisms, changes in the composition of the lipoproteins, affecting their interaction with enzymes involved in the intravascular processing of the particles and with specific receptors, and changes in the expressed activities of the enzymes that control this processing. At present there are a limited number of reports investigating the effects of dietary fatty acids on the intravascular processing of the lipoproteins and the mechanisms by which dietary fat quality and quantity affect the activities of the various enzymes involved in the interconversion and modifications of the lipoproteins involved in atherogenesis.

a. LPL. Studies in animal model systems indicate that the type of dietary fat affects plasma postheparin LPL activity in some species, whereas this effect is observed in other species only when dietary fat is combined with dietary cholesterol. Studies in rabbits (Van Heek and Zilversmit, 1990) indicate that postheparin LPL activity levels are similar in animals fed cholesterol-free diets containing 14% (by weight) coconut oil, olive oil, or corn oil. In contrast, when 0.5% cholesterol is added to the diets, LPL activity is significantly higher in rabbits fed the coconut oil diet compared to an olive oil-containing diet. These data are similar to those reported by Wang *et al.* (1987) from studies in cynomolgus monkeys fed low- and high-P:S diets (P:S 0.34 versus 2.2), which found no effect of fat quality on LPL activity.

In contrast, three studies in rats suggest that both the amount and the type of dietary fat modulate plasma postheparin LPL activity and that intake of

PUFA-rich diets increases LPL activity (Pawar and Tidwell, 1968; Coiffier *et al.*, 1987; Baltzell *et al.*, 1991). Baltzell *et al.* (1991) reported that feeding rats a PUFA corn oil diet (30% ene fat), as compared to a SFA lard diet, significantly increased soleus muscle LPL activity, whereas adipose tissue LPL was slightly, but not significantly, increased. Cryer *et al.* (1978) noted that guinea pigs fed 20% (by weight) corn oil had significantly higher levels of perirenal and epididymal LPL and similar levels of cardiac and skeletal muscle LPL compared to guinea pigs fed 20% (by weight) beef tallow. While the reports of both Baltzell *et al.* (1991) and Cryer *et al.* (1978) indicated higher levels of LPL in animals fed PUFA, there is a clear conflict as to the specificity of the effects in adipose and muscle.

Whether these contrasting results of dietary fat effects on LPL activity are due to species differences or to experimental design differences (dietary fat quantity and quality, *in vitro* substrates for LPL analysis, amount of dietary cholesterol, etc.) remains uncertain. Obviously there is a need for additional studies to address these questions.

Unfortunately, the data regarding dietary fat effects on plasma postheparin LPL activity in humans are contradictory and no clear conclusions regarding the effects of changes in dietary fat quality can be made. The available reports are conflicting in that two studies indicate LPL activity is increased (Bagdade *et al.*, 1970; Nestel and Barter, 1973) while two others find that LPL is unchanged (Chait *et al.*, 1974; Weintraub *et al.*, 1988) when PUFA-containing diets are compared to SFA diets under similar conditions. Such differences could be due to the range of dietary fat P:S ratios tested in different studies and different assay conditions for determining postheparin lipase activity. As noted by Weintraub *et al.* (1988), the fatty acid saturation of the substrate used for lipase activity measurements can have a significant effect on the rate of triacylglycerol hydrolysis. Miller *et al.* (1981) have shown that unsaturated glycerides are more readily hydrolyzed by lipases than are saturated glycerides, and that LPL has a lower affinity for monacylglycerols that contain a saturated fatty acid group. These specificity factors of LPL could be significant in determining the relative rates of hydrolysis of triacylglycerides in chylomicrons and VLDL during intake of diets containing SFA or PUFA.

b. HTGL. The present state of knowledge regarding dietary fat effects on HTGL activity is no better than our understanding of the effects of postheparin LPL activity, and again is limited to animal model studies. The data suggest that HTGL levels are increased with increased fat intake and that animals fed SFA have higher levels of HTGL than do rats fed PUFA. Coiffier *et al.* (1987) found that when rats were fed 14% (by weight) mutton tallow fat diets, HTGL levels were significantly higher than those found in animals fed 14% sunflower oil-containing diets. In a similar manner, Baltzell *et al.* (1991) found higher HTGL levels in rats fed 30% ene lard as compared to corn oil.

While these studies suggest that intake of PUFA lowers HTGL activity, studies in cynomolgus monkeys indicated no differences in HTGL activity in animals fed 42% ene fat diets of P:S 0.34 or 2.2 (Wang *et al.*, 1987); however, the results could be complicated by the high cholesterol content of the diets (200 mg/kcal). Studies of the effect of fat saturation on HTGL activity in humans suggest that intake of PUFA has no effect on activity compared to SFA intake (Weintraub *et al.*, 1988). It is apparent that additional studies are needed to define the effects of dietary fat quantity and quality on HTGL activity as well as the interactive effects of changes in enzyme activity coupled with changes in the fatty acid composition of chylomicron and VLDL remnants induced by fatty acids of varying degrees of unsaturation and chain lengths.

c. LCAT. Esterification of plasma-free cholesterol to cholesteryl ester is catalyzed by plasma LCAT, which is activated by apoA-I. Studies in both animal model systems and in humans indicate that plasma LCAT activity decreases when dietary SFA are replaced with PUFA. It has been shown in rats (Larking and Sutherland, 1977) and pigs (Forsythe *et al.*, 1980) that as the dietary P:S ratio increases, LCAT activity falls. Data from human studies indicate the same pattern, with LCAT activity declining as the dietary P:S ratio increases (Miller *et al.*, 1975; Gjone *et al.*, 1972; Wallentin, 1978a,b). Studies in animals and in humans suggest that the composition of HDL fatty acids can affect LCAT activity (Miller *et al.*, 1975; Larking and Sutherland, 1977) and suggest that intake of PUFA lowers not only plasma LCAT activity but also the ability of the lipoprotein substrates to support enzyme activity.

d. CETP. The transfer of HDL cholesteryl esters generated by the action of LCAT to apoB-containing lipoproteins, VLDL and LDL, is catalyzed by plasma CETP (Fielding, 1987), and studies have shown that CETP activity is regulated by a number of physiological factors (Quig and Zilversmit, 1990). CETP activity is thought to play an important role in reverse cholesterol transport by facilitating the transfer of cholesteryl esters from HDL to VLDL and LDL, thereby allowing plasma HDL particles to take up free cholesterol from extrahepatic tissues. Plasma CETP also plays an important role in the intravascular remodeling of plasma LDL in terms of LDL cholesteryl ester content.

Studies of dietary fat effects on plasma CETP activity are complicated by the wide range of CETP activity expressed by various animal models (Ha and Barter, 1982). Studies in the hamster have shown that plasma CETP activity is increased almost twofold by intake of SFA butter fat (15% by weight), as compared to PUFA margarine (Stein *et al.*, 1990). Addition of 2% cholesterol to either test diet resulted in an additional increase in plasma CETP-mediated transfer of cholesteryl ester and there was a positive correlation between plasma cholesterol levels and CETP activity. In a similar manner, studies in marmosets have shown

that plasma CETP activity is increased twofold to threefold when animals consume a 28% ene SFA and 0.2% cholesterol diet compared to a PUFA and low-cholesterol diet (Abbey *et al.*, 1990b). The observed increase in CETP activity of marmosets fed the atherogenic diet was evident whether measured in the $d > 1.21$ g/ml plasma fraction, indicative of increased CETP mass, or in the whole plasma, which incorporates effects of differences in the composition and concentrations of the donor and acceptor lipoproteins (Abbey *et al.*, 1990b). What remains unknown is whether the increased plasma cholesterol levels associated with the intake of SFA resulted from or caused the observed increase in CETP activity. In the marmoset study it is also unclear what part dietary cholesterol played in the observed increase, since these nonhuman primates are extremely sensitive to cholesterol.

The effects of dietary fat quality on plasma CETP activity in humans have been investigated by Groener *et al.* (1991), who reported that shifting patients from a SFA-containing diet (35% ene, P:S 0.24) to a PUFA diet (35% ene, P:S 1.01) did not affect plasma CETP activity, even though plasma total cholesterol levels were significantly reduced. These investigators noted a positive correlation between CETP activity and VLDL + LDL cholesterol levels, a negative correlation between CETP activity and plasma HDL cholesterol concentrations, and a positive correlation between changes in CETP activity and changes in plasma VLDL + LDL cholesterol during the dietary fat interventions. In contrast, intake of a diet containing MUFA resulted in significantly lower plasma CETP activity (Groener *et al.*, 1991).

The evidence indicates that the effects of dietary fatty acids on CETP activity relate to changes in plasma VLDL and LDL cholesterol levels and that under most conditions interventions that lower plasma levels of the apoB-containing lipoproteins lower plasma CETP activity. What remains uncertain is the cause and effect relationship: Does CETP activity decline because plasma VLDL and LDL levels decrease or do the levels of the lipoproteins decline in part because of lower CETP activity? Analysis of CETP mass and mRNA levels during dietary interventions modifying the amount and type of dietary fatty acids would provide valuable data addressing these questions (Quintet *et al.*, 1990).

As noted above, intake of PUFA-containing diets results in LDL particles with reduced cholesteryl ester content. Such changes in LDL composition could result from the combination of two factors, reduced CETP activity, thereby reducing the rate of cholesteryl ester transfer from HDL to LDL, and since LDL fractional catabolic rates are increased, a reduced LDL residence time in the plasma compartment, thereby decreasing the uptake of cholesteryl esters by LDL particles. In addition, studies have demonstrated that the rate of transfer of cholesteryl ester from HDL to LDL can be significantly affected by differences in LDL composition (Dullaart *et al.*, 1987). Compositional changes in VLDL and LDL induced by changes in dietary fat quality no doubt influence intravas-

cular processing of the particles. In theory, the combination of decreased plasma residence time and CETP activity could explain some of the observed LDL compositional changes during intake of a diet high in PUFA.

VI. PLASMA LIPID/LIPOPROTEIN RESPONSES TO MONOUNSATURATED FATTY ACIDS

In the early studies of the effects of dietary fats on plasma lipids, MUFA and carbohydrates were considered to have a neutral effect on plasma cholesterol levels and consequently were not considered important in estimates of dietary fat effects on plasma lipids. Subsequent studies have shown that intake of MUFA in the diet, whether as a substitute for SFA or PUFA calories or for carbohydrate calories, has unique effects on the plasma lipoprotein profile. Even though early studies by Keys *et al.* (1965) indicated that serum cholesterol levels fell almost 3 mg/dl for every 1% substitution of SFA with MUFA, the primary effect was considered to be due to removal of SFA, not addition of MUFA, since caloric substitution with carbohydrates achieved similar results. While this may in fact be the case when considering effects on plasma total cholesterol levels, evidence indicates that the effects on lipoprotein fractions are very different.

A. MONOUNSATURATED FATTY ACIDS AND PLASMA LIPOPROTEINS

Interest in the effects of dietary MUFA on plasma lipoproteins was renewed in 1985 when Mattson and Grundy (1985) reported that intake of oleic acid (C18:1) was as effective as linoleic acid (C18:2) in lowering LDL cholesterol levels and, more importantly, did not lower HDL cholesterol concentrations in normolipidemic patients (Fig. 5). In this study, total fat intake was maintained at 40% ene and the relative proportions of SFA, MUFA, and PUFA (S:M:P) shifted from 20:16:4 to 3:29:7 to 5:6:29. Plasma LDL cholesterol levels were decreased by 30 mg/dl (-17%) by the high-MUFA and high-PUFA diets, while HDL cholesterol levels were reduced 5 mg/dl (-10%) only when the high-PUFA diet was tested (Mattson and Grundy, 1985). Since publication of this report on the effects of MUFA on specific lipoprotein fractions, there has been extensive research directed at determining the potential role of MUFA as part of a plasma cholesterol-lowering diet. In addition, studies have been carried out investigating the relative benefits of a low-fat, PUFA diet versus a moderate-fat, MUFA diet in lowering plasma LDL levels, yet maintaining plasma HDL concentrations (Table VI).

Sirtori *et al.* (1986) investigated the effects of a low-fat diet (28% ene) containing either corn oil or olive oil on plasma lipids, lipoprotein, and apoproteins

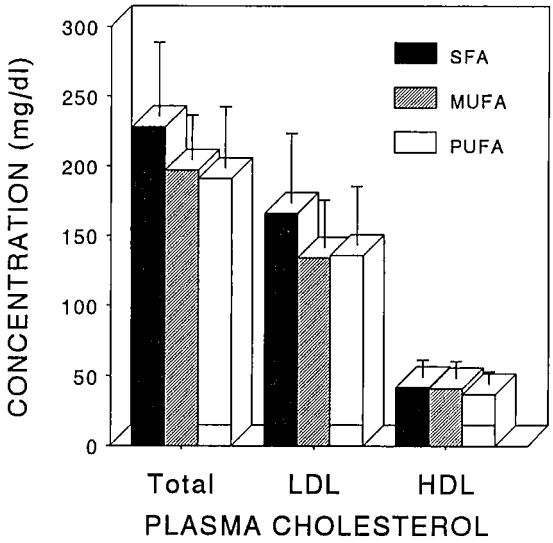

FIG. 5. Comparison of the effects of intake of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) on plasma lipid and lipoprotein levels. Subjects (N = 20) were fed liquid formula diets containing 40% ene fat either high in SFA (P:M:S = 0.20:0.81:1.0), MUFA (P:M:S = 2.08:8.54:1.0), or PUFA (P:M:S = 6.55:1.31:1.0) for 4 weeks, after which plasma lipids and lipoproteins were determined. Plasma total and LDL cholesterol levels were significantly lower ($P < 0.05$) on both the MUFA and PUFA diets compared to the SFA phase; HDL cholesterol was significantly lower on the PUFA diet. (After Mattson and Grundy, 1985.)

in 23 hypercholesterolemic subjects. The data indicated that both diets had similar lowering effects on plasma total and LDL cholesterol and apoB levels, while intake of olive oil had less of a lowering effect on plasma HDL cholesterol and apoA-I levels. This study also found that the plasma apoA-I:apoB ratio was significantly higher on the olive oil diet, which is indicative of a less atherogenic lipoprotein profile (Sirtori *et al.*, 1986).

Grundy (1986) compared the effects on plasma lipoprotein levels of two high-fat formula diets (40% ene), one high in SFA and one high in MUFA, relative to a low-fat diet (20% ene) with a P:M:S ratio of 1:1:1. Both the high-MUFA diet and the low-fat diet significantly reduced plasma total and LDL cholesterol levels compared to the high-SFA diet; however, while the high-MUFA diet had no effect on plasma triacylglycerol and HDL levels, intake of the low-fat diet raised triacylglycerol levels and lowered HDL concentrations. The LDL:HDL ratio was significantly lower on the MUFA diet compared to the low-fat diet (Grundy, 1986), consistent with a less atherogenic plasma lipoprotein profile during intake of the MUFA diet. In a subsequent report, Grundy *et al.* (1988)

repeated these studies using solid food diets rather than formula feeding and again observed that intake of MUFA lowered LDL, but not HDL, cholesterol levels, whereas intake of a low-fat diet resulted in significant reductions in both LDL and HDL.

Similar results have been reported by Mensink and Katan (1987) from a study of 48 men and women fed test diets rich in either complex carbohydrates (22% ene fat) or olive oil (40% ene fat), which showed that intake of the olive oil-rich diet, unlike the low-fat, carbohydrate-rich diet, caused specific reductions in non-HDL cholesterol with serum triacylglycerol levels unchanged. These data raise the question of whether a low-fat (30% ene; P:M:S ratio of 1:1:1) or a moderate-fat diet (35% ene) high in MUFA is more effective for altering the plasma lipoprotein profile and CHD risk (Grundy, 1989).

The effect of a moderate-fat (39% ene), MUFA-rich diet versus a low-fat (29% ene) diet on plasma HDL subfractions was investigated in 11 young males by Baggio *et al.* (1988). Plasma total and LDL cholesterol, triacylglycerol, and apoB levels were significantly lower on the MUFA diet; however, total, HDL_2, and HDL_3 cholesterol and apoA-I levels and the LDL:HDL ratio were similar. Whether this was due to the low PUFA content of the low-fat (P:M:S = 4:13:12) and MUFA (P:M:S = 4:25:10) diets is unclear.

In a study of 48 normolipidemic men and women, Mensink *et al.* (1989) compared the relative effects of changing from a SFA (38% ene fat) diet to either a low-fat diet (22% ene) or a MUFA (40% ene fat) diet. Both diets lowered plasma total and LDL cholesterol levels; however, intake of the low-fat diet also resulted in increased plasma triacylglycerol concentrations and lower levels of total HDL and HDL_3 cholesterol and plasma apoA-I and apoA-II levels. Compared to the high-SFA diet phase, the ratio of apoA-I:apoB, an indicator of reduced atherosclerotic risk, was increased during intake of the MUFA diet, whereas it was decreased in subjects on the low-fat, high-carbohydrate diet (Mensink *et al.*, 1989). The data indicate that neither VLDL nor HDL behave neutrally when dietary oleic acid and carbohydrates are exchanged, and that one possible reason that this was not noted previously is that when total plasma cholesterol levels are measured, the changes in VLDL + LDL and HDL cholesterol are of similar mass amounts, but in opposite directions.

Many studies of the effectiveness of MUFA in lowering plasma total and LDL cholesterol levels have compared intakes of low-fat and moderate-fat, oleic acid-rich diets. To address the question of whether MUFA are as effective as PUFA in altering plasma lipoproteins levels, Mensink and Katan (1989) studied men and women fed diets of similar fat quantity (36% ene) but varying in the relative proportions of SFA, MUFA, and PUFA. All subjects (n = 58) were initially fed a SFA-rich diet (P:M:S = 5:12:19) and then switched to either a MUFA (8:15:13) or a PUFA (13:11:13) diet. The selection of these dietary fat mixtures was designed to mimic the types of dietary changes being recommended by

TABLE VI

STUDIES OF DIETARY MONOUNSATURATED FATTY ACID EFFECTS ON PLASMA LIPOPROTEINS

SFA	MUFA	PUFA	Total cholesterol (mg/dl)	LDL-C (mg/dl)	HDL-C (mg/dl)	Total triacylglycerol[a] (mg/dl)	Reference
20	16	4	133 ± 21	81 ± 13	40 ± 11	72 ± 35	Becker *et al.* (1983)
4	32	4	127 ± 13	71 ± 18	43 ± 9	75 ± 34	(n = 12)
4	16	20	123 ± 16	65 ± 15	45 ± 9	70 ± 35	
20	16	4	228 ± 53	166 ± 50	42 ± 12	143 ± 44	Mattson and
3	29	7	197 ± 32	134 ± 34	41 ± 12	134 ± 42	Grundy (1985)
5	10	29	191 ± 44	136 ± 42	37 ± 9	133 ± 49	(n = 12)
8	10	10	239 ± 16	175 ± 14	41 ± 2	146 ± 17	Sirtori *et al.* (1986)
7	18	3	246 ± 18	181 ± 15	42 ± 2	152 ± 18	(n = 23)
25	8	7	254 ± 10	178 ± 10	46 ± 3	177 ± 23	Grundy (1986)
4	28	8	220 ± 19	146 ± 8	43 ± 2	176 ± 15	(n = 7)
7	7	7	237 ± 13	162 ± 14	32 ± 2	240 ± 41	
18	16	4	228 ± 17	163 ± 14	36 ± 5	160 ± 24	Grundy (1986)
4	28	8	187 ± 7	119 ± 10	34 ± 4	182 ± 29	(n = 4)
7	7	7	199 ± 5	123 ± 9	30 ± 4	227 ± 37	
7	27	5	208 ± 10	140 ± 8	50 ± 4	104 ± 9	Grundy *et al.* (1988)
7	7	6	208 ± 7	139 ± 5	46 ± 4	113 ± 8	(n = 10)
12	13	4	224 ± 31	159 ± 33	50 ± 6	83 ± 21	Baggio *et al.* (1988)
10	25	4	204 ± 25	139 ± 25	50 ± 6	62 ± 23	(n = 11)
19	12	5	199 ± 34	128 ± 29	53 ± 12	88 ± 43	Mensink and Katan
13	15	8	171 ± 34	105 ± 26	50 ± 12	83 ± 38	(1989) (n = 29)

19	12	5	198 ± 28	129 + 26	55 ± 13	76 ± 29	Mensink and Katan
13	11	13	178 ± 26	111 ± 23	52 ± 15	73 ± 34	(1989) (n = 29)
21	14	5	244 ± 6	177 ± 6	43 ± 2	146 ± 12	Wardlaw and Snook
7	28	6	203 ± 6	140 ± 6	43 ± 2	132 ± 9	(1990) (n = 20)
8	14	19	191 ± 6	132 ± 5	42 ± 2	115 ± 10	
14	15	7	173 ± 18	115 ± 23	55 ± 12	91 ± 36	McDonald *et al.*
5	20	10	142 ± 13	87 ± 16	50 ± 10	73 ± 24	(1989) (n = 8)
7	7	22	142 ± 11	85 ± 14	49 ± 9	73 ± 24	
8	9	10	184 ± 35	125 ± 39	46 ± 11	86 ± 47	Dreon *et al.* (1990)
8	14	5	185 ± 35	121 ± 33	45 ± 12	95 ± 52	(n = 34)
12	6	12	151 ± 22	84 ± 17	54 ± 11	69 ± 10	Chang and Huang
5	19	5	155 ± 22	90 ± 19	50 ± 7	83 ± 8	(1990) (n = 8)
8	16	8	136 ± 10	80 ± 9	41 ± 2	74 ± 9	Berry *et al.* (1991)
7	6	16	129 ± 10	74 ± 9	41 ± 2	70 ± 7	(n = 26)
15	14	9	207 ± 6	141 ± 6	43 ± 2	—	Wardlaw *et al.* (1991)
7	22	11	189 ± 6	124 ± 6	44 ± 4	—	(n = 16)
15	14	9	209 ± 7	146 ± 7	41 ± 2	—	Wardlaw *et al.* (1991)
7	9	22	178 ± 5	117 ± 5	39 ± 2	—	(n = 16)
7	19	4	215 ± 10	138 ± 10	58 ± 2	89 ± 9	Sola *et al.* (1990)
7	10	13	210 ± 10	132 ± 9	61 ± 1	79 ± 9	(n = 12)
7	10	13	194 ± 6	114 ± 9	67 ± 1	62 ± 4	
16	10	4	258 ± 13	171 ± 13	69 ± 4	88 ± 11	
14	14	5	170 ± 2	115 ± 2	44 ± 1	92 ± 3	Chan *et al.* (1991)
7	19	8	140 ± 4	90 ± 4	45 ± 1	65 ± 5	(n = 8)
6	19	9	139 ± 3	86 ± 4	43 ± 1	84 ± 5	
7	9	18	143 ± 3	94 ± 4	45 ± 1	70 ± 5	
7	10	17	140 ± 4	90 ± 4	44 ± 1	67 ± 5	

[a]To convert mg/dl triacylglycerol to mmol/liter, multiply by 0.01128.

health agencies. Plasma total and LDL cholesterol and apoB levels were equally reduced by both of the unsaturated fatty acid diets. The authors concluded that, as far as lipoprotein levels are concerned, it is immaterial whether SFA in the diet are replaced by a mixture of MUFA and PUFA or by PUFA alone; both diets lower LDL cholesterol and apoB levels and have similar effects on HDL cholesterol and apoA-I levels (Mensink and Katan, 1989). The authors do provide an important caveat in their conclusion in that it only holds true as long as extremely large amounts of PUFA, greater than 13% ene, are avoided. This may in part explain the differences between this study and the results reported by Mattson and Grundy (1985), wherein PUFA intake was at 29% ene and significant lowering of HDL cholesterol in response to the high-PUFA intake was observed.

This cannot, however, be the only explanation in that Wardlaw and Snook (1990) fed 20 men diets of similar fat quantity (40% ene) but dissimilar fat quality (P:M:S of 5:14:21 versus 6:28:7 versus 19:14:8) and reported that both unsaturated fatty acid-enriched diets had similar lowering effects on plasma total and LDL cholesterol and apoB levels, while HDL cholesterol and apoA-I levels were unaffected. These investigators concluded that MUFA and PUFA were equally effective substitutes for SFA in the diet when attempting to lower plasma LDL cholesterol levels. Similar findings have been reported by Becker *et al.* (1983), McDonald *et al.* (1989), Dreon *et al.* (1990), and Wardlaw *et al.* (1991), which indicate similar plasma lipid, lipoprotein, and apoprotein responses to intake of MUFA and PUFA when total dietary fat calories are kept constant.

In contrast, some recent studies suggest that when the diets are varied under more modest conditions, which maintain either a constant SFA intake or the P:S ratio, PUFA have a greater plasma total and LDL cholesterol lowering effect than do MUFA. Chang and Huang (1990) fed two diets of identical P:S ratio but differing in the P:M:S ratio (12:6:12 versus 5:19:5) and found that LDL cholesterol levels were significantly lower on the PUFA-rich diet even though SFA intake was higher. In a similar manner, Berry *et al.* (1991) tested 26 subjects on diets containing a constant percentage of SFA and exchanged the MUFA and PUFA (P:M:S of 8:16:8 versus 16:6:7) and observed lower plasma total and LDL cholesterol levels on the PUFA diet; HDL levels were unchanged.

Ginsberg *et al.* (1990) investigated the plasma lipoprotein responses to intakes of a typical American diet (38% ene fat, P:M:S of 17:12:10) versus a Step I diet (30% ene fat, P:M:S of 9:11:11) with and without addition of 7% ene MUFA. Plasma total and LDL cholesterol levels were significantly lower on the Step I and MUFA-enriched Step I diets. Although the HDL cholesterol levels were lower on the Step I diet compared to the average American diet or the MUFA-enriched Step I diet, the differences were not significant. These authors concluded that addition of MUFA to a Step I diet does not have a beneficial or detrimental affect on the efficiency of the diet in terms of plasma lipoprotein reductions.

The data from numerous studies clearly demonstrate that substitution of SFA

in the diet with MUFA results in lower plasma LDL cholesterol levels and, when done isocalorically, maintains plasma HDL cholesterol levels. The interpretation of many of the studies is confusing, because it depends on whether the MUFA are replacing SFA or PUFA, whether total fat calories remain constant, and the comparison level of PUFA in the diet. As discussed in detail below, many studies indicate that when fat calories are reduced, plasma HDL cholesterol levels are lowered. As discussed above, the data indicate that very high P:S diets result in lower plasma HDL cholesterol levels. What the data suggest is that the plasma LDL cholesterol-lowering effect is comparable whether SFA are replaced with PUFA or MUFA and that any observed changes in HDL levels usually result from a reduction in fat calories and/or extremely high intakes of PUFA. As known since the original studies of Keys *et al.* (1965) and Hegsted *et al.* (1965), SFA calories have the predominant fatty acid effect on plasma cholesterol levels and when they are replaced in the diet there is a reduction in plasma LDL cholesterol levels irrespective of the caloric substitute.

B. MECHANISM OF PLASMA LDL CHOLESTEROL LOWERING BY MONOUNSATURATED FATTY ACIDS

There have been no studies of the effects of substituting MUFA for either SFA or carbohydrate calories on *in vivo* lipoprotein metabolism in human subjects. With the absence of available human data defining the hypocholesterolemic mode of action of MUFA, potential mechanisms have been proposed from studies in animal model systems. There is, however, a fundamental difference between rodents and humans in terms of the plasma cholesterol response to dietary exchange for MUFA in that most rodent animal models do not exhibit a hypocholesterolemic response; in contrast, the data indicate that intake of olive oil-rich diets by rats, hamsters, guinea pigs, and rabbits elicits a hypercholesterolemic response. As discussed below, the responses of nonhuman primate models appear to reflect more closely the human responses.

Compared to dietary PUFA, intake of MUFA in rats results in increased levels of plasma and hepatic cholesterol levels (Kris-Etherton *et al.*, 1984). Similar findings have been reported for the rat (Beynen, 1987), guinea pig (Fernandez and McNamara, 1989; Fernandez *et al.*, 1990, 1992), rabbit (Beynen *et al.*, 1987), and hamster (Spady and Dietschy, 1988). Interestingly, intake of olive oil, compared to PUFA intake, has been shown to significantly reduce hepatic sterol synthesis, measured *in vivo* from the incorporation of ^3H_2O into sterols (Spady and Dietschy, 1988; Fernandez *et al.*, 1990), and by analysis of hepatic microsomal hydroxymethylglutaryl-CoA (HMG-CoA) reductase activity (Fernandez *et al.*, 1990, 1992). Sterol balance studies have shown that guinea pigs fed a 35% ene olive oil-enriched diet have a reduced fecal excretion of bile

acids, suggesting decreased catabolism of cholesterol (Fernandez *et al.*, 1990). Studies in the hamster (Spady and Dietschy, 1988) indicate that when cholesterol is added to an olive oil-containing test diet, hepatic cholesteryl ester content is increased six to nine times that found for the same amounts of cholesterol added to either a safflower oil- or coconut oil-containing diet. Similar results have been found in guinea pigs fed olive oil-containing diets with added cholesterol (E.C.K. Lin, M.L. Fernandez, and D.J. McNamara, unpublished observations).

Animal studies indicate that the lack of a hypocholesterolemic response to MUFA intake is due to the fact that MUFA intake has no effect on apoB/E receptor-mediated LDL catabolism. *In vitro* binding studies have shown that hepatic receptor number is the same for guinea pigs fed either 19% ene or 30% ene lard- or olive oil-containing diets (Fernandez and McNamara, 1989, 1991a), that the rate of hepatic clearance of plasma LDL in the hamster is the same for animals fed 20% (by weight) coconut oil or olive oil (Spady and Dietschy, 1988), and that the *in vivo* LDL fractional catabolic rate is similar for guinea pigs fed 30% ene olive oil or lard (Fernandez *et al.*, 1992).

There are only limited data available from studies in nonhuman primates investigating the effects of MUFA intake on lipoprotein metabolism. To date, only the African green monkey has been shown to have a hypocholesterolemic response to intake of MUFA, and in these studies the diets contained relatively high amounts of cholesterol (Rudel *et al.*, 1990). African green monkeys were fed 35% ene fat diets of varying P:M:S ratios (5:13:16, 7:25:3, or 27:4:3) containing 0.8 mg cholesterol/kcal and the plasma lipoprotein and apoprotein profiles were determined. Both unsaturated fatty acid diets lowered plasma total and LDL cholesterol and apoB levels compared to the SFA diet; however, intake of the PUFA diet also lowered plasma HDL cholesterol and apoA-I levels. As noted in many human studies, intake of MUFA has little effect on the plasma concentration of either HDL cholesterol or apoA-I.

Based on animal model studies it would seem that intake of MUFA does not affect LDL receptor expression. If the same is true for humans (an assumption with obvious limitations), then one could postulate that the hypocholesterolemic effect of MUFA intake relates to changes in LDL apoB production rates rather than to alterations in receptor-mediated LDL catabolism. Such a concept could be argued based on the observation that intake of PUFA lowers LDL production rates and increases the fractional catabolic rates of LDL without altering hepatic cholesterol metabolism. In contrast, intake of MUFA lowers hepatic cholesterol synthesis and catabolism and could in theory reduce LDL apoB production rates either by decreasing VLDL production or by direct synthesis of LDL. Clinical studies of the effects of MUFA intake on VLDL and LDL kinetics are needed to test the potential of this hypothesis and to define the metabolic effects of dietary MUFA.

C. MONOUNSATURATED FATTY ACIDS AND ATHEROSCLEROSIS

Considering the evidence for a plasma LDL cholesterol-lowering response to intake of MUFA, it could be argued that the hypocholesterolemic effect of diets high in MUFA foods (for example, olive oil) explains the low CHD mortality rates in Mediterranean countries (Keys *et al.*, 1986). In view of the plasma cholesterol levels in some of these populations this cannot be the only explanation, and recent studies suggest that it may relate to the relative susceptibility of the oleic acid-enriched LDL particle to oxidative stress. Studies indicate that oxidative modification of LDL results in generation of modified LDL particles with enhanced atherogenic potential (Steinberg *et al.*, 1989). Parthasarathy *et al.* (1990) demonstrated that LDL isolated from rabbits fed diets high in oleic acid were resistant to oxidative modification as compared to LDL from animals fed diets high in linoleic acid-containing sunflower oil. The authors suggested that diets containing high levels of oleic acid might reduce the progression of atherosclerosis by two mechanisms, a lowering of plasma LDL concentrations and production of an LDL particle resistent to oxidative modification (Parthasarathy *et al.*, 1990). This hypothesis has been extended to humans by Berry *et al.* (1991), who demonstrated that LDL isolated from patients on a high-MUFA diet were less susceptible to oxidative stress than LDL from patients fed PUFA-containing diets. The hypothesis of reduced oxidative potential of oleic acid-enriched LDL could in part explain why there is less atherogenicity in rabbits fed olive oil plus cholesterol diets compared to corn oil plus cholesterol diets, even though the plasma lipoprotein cholesterol concentrations were similar (Leth-Espensen *et al.*, 1988).

In contrast to the cross-cultural epidemiology data and the findings of clinical diet trials, results from the Framingham Study indicated a significant positive association between the 16-year incidence of CHD and the proportion of calories derived from total fat and MUFA in a male cohort aged 45 to 55 years (Posner *et al.*, 1991). The authors suggest that since a large proportion of MUFA in the American diet is derived from animal products, as opposed to intake of olive oil in Mediterranean countries, this would be associated with higher intakes of SFA; however, CHD incidence was not significantly associated with SFA intake in this cohort. Whether such findings relate to the vagaries of dietary fat intake data obtained from 24-hour recalls or whether they actually represent a relationship to total fat intake, of which a large percentage is MUFA, remains to be determined.

The potential role of MUFA-containing moderate-fat diets as part of a plasma LDL cholesterol-lowering dietary regimen, with the added potential of reducing LDL oxidation, is an exciting and active research area that deserves attention. Clinical studies to define the mechanisms of action of MUFA on LDL metabolism, both *in vivo* and *in vitro,* are essential and long overdue. Without data

on the mechanisms of action of MUFA intake on lipoprotein metabolism, and considering the contradictory epidemiological data, it is probably premature to enter into debates regarding the relative merits of low-fat versus MUFA-rich moderate-fat diets as plasma cholesterol-lowering, CVD risk-reducing interventions. More detailed information needs to be obtained regarding the metabolic effects of MUFA on plasma lipoproteins before changes in dietary patterns can be reasonably argued.

D. TRANS FATTY ACIDS

While there is convincing evidence that dietary substitution of SFA with MUFA lowers plasma LDL cholesterol levels but maintains HDL cholesterol concentrations, this applies to cis MUFA. The situation with trans MUFA is less clear and is the topic of some debate. Early studies indicated that intake of elaidic acid (C18:1 trans), the major trans fatty acid found in the American diet, had no effect on plasma cholesterol levels and could be considered neutral, similar to oleic acid (C18:1 cis), in terms of the plasma cholesterol response (Erickson *et al.*, 1964; Mattson *et al.*, 1975; Laine *et al.*, 1982). Recent studies by Mensink and Katan (1990) indicate that intake of elaidic acid significantly increases plasma LDL cholesterol and apoB levels while lowering HDL cholesterol and apoA-I plasma concentrations. In these studies, 34 women and 25 men were fed diets containing 40% ene fat, with the sole variable being 10% ene of either oleic acid, trans isomers of oleic acid (elaidic acid), or SFA. Each diet was fed for 3 weeks in a random order. Plasma LDL cholesterol and apoB levels were highest on the SFA diet; however, LDL cholesterol and apoB concentrations were significantly higher during intake of the trans fatty acid-rich diet compared to the oleic acid-containing diet. Of greater significance was the finding that while HDL cholesterol and apoA-I levels were similar during intake of the cis MUFA and SFA diets, both HDL cholesterol and apoA-I decreased during intake of the trans fatty acids. The authors concluded that the plasma lipoprotein response to dietary trans fatty acids was unfavorable in that LDL levels increased and HDL concentrations decreased, resulting in less desirable ratios of LDL:HDL and of apoA-I:apoB (Mensink and Katan, 1990).

One hypothesis for the observed effects of trans MUFA on plasma lipoprotein levels is that due to their structural similarity to SFA, trans fatty acids increase plasma cholesterol levels (Grundy, 1990); however, this does not explain the HDL-lowering effect observed in the studies of Mensink and Katan (1990). Considering the increased use of hydrogenated fatty acids as a substitute for tropical oils in commercial food products, this area of investigation requires clarification to address whether dietary trans fatty acids in the range of intake found in the United States, 8–10 g/day, has a significant effect on the plasma lipoprotein concentrations and distribution.

Animal studies of the effects of trans fatty acids on lipoprotein concentrations,

composition, structure, and atherogenicity have not found evidence for a significant effect of trans MUFA on any of these parameters (Jackson *et al.*, 1977; Toda *et al.*, 1985), suggesting that metabolic differences between species could be confounding our ability to investigate mechanisms of action and differences between cis and trans fatty acids.

Based on the current state of our understanding of the effects of trans fatty acids on plasma lipids and lipoproteins, it is probably premature to make recommendations regarding their intake and role in CHD risk; however, it would probably be prudent for those attempting to reduce an elevated plasma LDL cholesterol level, especially if plasma HDL levels are low, to minimize intake of trans MUFA until a better understanding of their effects on plasma lipoproteins is available.

VII. DIETARY FAT EFFECTS ON PLASMA LIPOPROTEIN LEVELS: QUANTITY VERSUS QUALITY

There are a number of uncertainties regarding the relative benefits of changes in dietary fat quality versus reductions in fat quantity as the modality to reduce plasma cholesterol levels and CHD risk (Grundy, 1989). The confusion deals with which diet is more effective in reducing a hyperlipidemic plasma profile, a 30% ene low-fat diet with a P:M:S ratio of 1:1:1, or a more moderate-fat diet with an increased level of MUFA. The quandary deals with which diet is best at lowering atherogenic LDL cholesterol levels without the potential effects of lowering plasma HDL cholesterol concentrations and increasing triacylglycerol levels. This debate gains practical importance when recommendations are made by some health professionals, i.e., that dietary fat intakes be reduced to 20% of calories, or lower. The question of whether there are health risks associated with very low-fat diets has been raised (Reaven, 1986; Grundy, 1989; Crouse, 1989) and deserves appropriate consideration in the development of population-based dietary recommendations. Some of the concerns expressed regarding implementation of low-fat, high-carbohydrate diets include hypertriglyceridemia, hyperglycemia, hyperinsulinemia, and decreased HDL cholesterol, all of which have the potential to increase CVD risk (Reaven, 1986; Grundy, 1989). As noted above regarding studies of the potential benefits of MUFA intake on plasma lipoprotein levels, reductions in LDL cholesterol without lowering HDL cholesterol are readily achieved when the percentage of fat calories remains constant, whereas plasma HDL levels are usually reduced when fat calories are decreased. The amount of dietary fat can play as major a role in determining plasma lipoprotein levels, as does the type of dietary fat.

Early studies of the effects of dietary factors on plasma cholesterol levels indicated that dietary carbohydrates had a neutral effect on plasma cholesterol levels (McGandy *et al.*, 1966) and any related cholesterol-lowering response to

a low-fat, high-carbohydrate diet was attributed to a decrease in calories from SFA, not an increase in calories from carbohydrates. In contrast, studies by Ahrens *et al.* (1961) showed that subjects with elevated plasma triglycerides exhibited even higher plasma triacylglyceride levels when fed a high-carbohydrate diet, a phenomenon termed "carbohydrate-induced lipemia." Studies in the 1960s and 1970s investigated and debated the existence of carbohydrate-induced hypertriglyceridemia and whether this response was a common effect of high-carbohydrate diets or due to acute exposure to certain types of sugars. While these studies determined the effects of dietary fat quantity on plasma total cholesterol and triglyceride levels, it is now clear that variations in dietary fat calories, with or without changes in fat quality, can have unique effects on plasma lipoprotein fractions, the composition and metabolic characteristics of specific lipoproteins, and, in many cases, the magnitude of the responses of the lipoproteins related to the extent of endogenous triacylglyceridemia expressed by subjects in any given study.

A. FAT QUANTITY AND CHYLOMICRON LEVELS

As total dietary fat intake is reduced, production of chylomicrons for exogenous fat transport is correspondingly lowered, and vice versa. The reduction in chylomicron production also results in a reduction in plasma levels of chylomicron remnants, which, if postprandial chylomicrons and their remnant particles are atherogenic in normolipidemic individuals (Weintraub *et al.*, 1987a,b), would in theory reduce CVD risk. The atherogenicity of postprandial triacylglycerol-rich particles in normolipidemic individuals has yet to be proved, and, as such, the possible benefits of a low-fat, high-carbohydrate diet via this mechanism remain speculative.

Nestel and Barter (1973) reported that rates of clearance of intravenously infused triacylglycerols (Intralipid) were similar for patients on 65% ene SFA intake (P:S 0.25) compared to zero fat intake, and that both clearance rates were lower than that of patients on 65% ene PUFA intake (P:S 4.0). The authors concluded that dietary fat quantity had no unique effect on *in vivo* rates of triacylglycerol clearance, whereas dietary fat type did have a significant effect. To date there is no evidence that intake of low-fat diets, under conditions wherein fat quality remains constant, has any unique metabolic effects on chylomicron production, transport, or catabolism.

B. FAT QUANTITY AND VLDL LEVELS

A consistent finding of most studies investigating the effects of high-fat versus low-fat diets on plasma VLDL triacylglycerol levels is that VLDL levels increase during intake of low-fat, high-carbohydrate diets (Table VII). The data indicate

TABLE VII

DIETARY FAT QUANTITY AND PLASMA LIPID AND LIPOPROTEIN LEVELS

Fat (% ene)[a]				Plasma cholesterol (mg/dl)			Triglyceride (mg/dl)	Reference
Total	S	M	P	Total	LDL	HDL		
38	19	10	10	255 ± 16	184 ± 16	47 ± 3	149 ± 15	Wolf and Grundy
29	14	7	7	239 ± 13	166 ± 16	44 ± 2	171 ± 33	(1983) (n = 7)
48	26	20	2	254 ± 31	155 ± 25	87 ± 25	62 ± 23	Lukaski *et al.*
30	16	12	2	243 ± 32	158 ± 34	68 ± 17	79 ± 20	(1984) (n = 3)
42	13	16	13	194 ± 40	135 ± 38	49 ± 8	64 ± 18	Weisweiler *et al.*
32	10	12	10	193 ± 28	142 ± 33	48 ± 9	75 ± 23	(1985) (n = 22)
30	10	10	10	175 ± 9	118 ± 8	39 ± 3	123 ± 9	Grundy *et al.* (1986)
20	7	7	7	170 ± 8	114 ± 8	33 ± 2	151 ± 17	(n = 9)
38	18	12	6	188 ± 12	—	53 ± 3	38 ± 3	Jones *et al.* (1987)
19	8	6	3	176 ± 12	—	46 ± 2	55 ± 5	(n = 16)
39	11	14	11	182 ± 9	—	55 ± 2	45 ± 2	Jones *et al.* (1987)
19	5	7	5	167 ± 12	—	55 ± 4	54 ± 6	(n = 16)
39	7	27	5	208 ± 10	140 ± 8	50 ± 4	104 ± 9	Grundy *et al.* (1988)
20	7	7	6	208 ± 7	139 ± 5	46 ± 4	113 ± 8	(n = 10)
38	10	25	4	204 ± 25	139 ± 25	50 ± 6	62 ± 23	Baggio *et al.* (1988)
28	12	13	4	224 ± 31	159 ± 33	50 ± 6	83 ± 21	(n = 11)
40	15	19	6	232 ± 15	161 ± 16	43 ± 4	213 ± 38	Ullman *et al.* (1991)
35	11	16	8	223 ± 15	144 ± 16	41 ± 3	232 ± 37	(n = 8)
30	8	14	8	216 ± 15	141 ± 16	44 ± 4	237 ± 30	
25	6	11	8	209 ± 14	134 ± 14	42 ± 3	230 ± 37	
20	5	7	8	198 ± 13	126 ± 15	36 ± 3	230 ± 35	

[a]Fats are saturated (S), monounsaturated (M), and polyunsaturated (P); ene, energy percent.

that increases in plasma VLDL triacylglycerol levels in response to high-carbohydrate intake occur in both normolipidemic and hypertriglyceridemic patients.

There have been a number of studies investigating the effects of dietary fat quantity on VLDL apoB and triacylglycerol metabolism in human subjects. Analyses of plasma VLDL kinetics have shown that when dietary fat calories are decreased from 40–45% to 15–25% and dietary fat quality and cholesterol remain constant, VLDL apoB production rates are increased by 31% and the fractional catabolic rate is decreased by 18% (Nestel *et al.*, 1979; Cortese *et al.*, 1983; Huff and Nestel, 1982). One observation noted in all studies of the effects of fat quantity on VLDL apoB kinetics has been the high degree of variation in individual responses, suggesting that some patients are more sensitive to the

effects of a low-fat diet than others. Irrespective of the variability, the data demonstrate that the combined effects of increased VLDL apoB production and decreased catabolism can account for increased plasma VLDL triacylglycerol concentrations during intake of high-carbohydrate diets.

Studies by Abbott *et al.* (1990) in seven nondiabetic Pima Indians fed diets of either 42% ene fat (P:S 0.29) or 21% ene fat (P:S 1.0) showed that intake of a low-fat diet resulted in a slight increase in plasma VLDL apoB and triacylglycerol levels and no changes in VLDL apoB production or fractional catabolic rates. There was also no change in VLDL triacylglycerol production or catabolism rates upon shifting from the high-fat, low-P:S to the low-fat, high-P:S diet. These investigators did find that the low-fat diet decreased the rate of conversion of VLDL to IDLs and to LDL with a corresponding increase in the rate of VLDL apoB direct removal (Abbott *et al.*, 1990). The data suggest that the high-carbohydrate diet resulted in a decreased rate of VLDL triacylglycerol removal involved in the delipidation cascade sequence of VLDL conversion to IDLs and LDL, facilitating increased direct removal of the VLDL. What is uncertain are the specific effects of changes in dietary fat P:S ratio versus changes in fat quantity on the parameters of VLDL metabolism measured in this study (Abbott *et al.*, 1990).

Studies by Melish *et al.* (1980) and Ginsberg *et al.* (1981) have shown that when dietary fat is reduced from 40% of calories to zero, VLDL apoB catabolic rates are significantly reduced, the proportion of VLDL undergoing direct catabolism versus conversion to LDL is significantly reduced, and the fraction directly removed from the plasma compartment is increased (Ginsberg *et al.*, 1981). These data are similar to those of Abbott *et al.* (1990) and suggest that whether the P:S ratio of the diet is changed when fat intake is reduced or the fat calories are reduced to nearly zero, the fraction of plasma VLDL converted to LDL is decreased while the fraction involved in direct removal is increased. As discussed in detail below, the effects of a high-carbohydrate diet on VLDL composition and apoE content (Keidar *et al.*, 1990) could in part explain the observed changes in VLDL metabolic channeling.

In contrast to the VLDL apoB kinetic data, analysis of VLDL triacylglycerol kinetics indicated that a shift from 40% ene to zero fat increased VLDL triacylglycerol production rates while having no effect on the fractional catabolic rate (Melish *et al.*, 1980). This disassociation of VLDL apoB and triacylglycerol kinetics indicates that changes in VLDL triacylglycerol production need not be associated with a corresponding change in VLDL apoB production. In part, the difference in observed rates of VLDL apoB and triacylglycerol production can be explained by the observation that high-carbohydrate diets cause secretion of increased numbers of large triacylglycerol-enriched VLDL particles by the liver (Witztum and Schonfeld, 1978), resulting in an increased ratio of VLDL triacylglycerol to apoB (Melish *et al.*, 1980). What remains unclear are the mechanisms involved in the differences in fractional catabolic rates of VLDL apoB

and triacylglycerol (Melish *et al.*, 1980; Abbott *et al.*, 1990). Such an observation could be due to differences in the triacylglycerol content of the VLDL particles, changes in exchange rates of VLDL triacylglycerol with HDL, effects on lipolytic enzymes involved in the delipidation cascade, and the high degree of interindividual variability in response to the change from a high-to a low-fat diet.

Studies by Keidar *et al.* (1990) in a group of normolipidemic subjects of the effects of a high-carbohydrate diet on VLDL composition, subfraction distribution, and apoB-100 epitope expression have shown that when fat calories are reduced from 40% ene to less than 1%, VLDL triacylglycerol and cholesterol levels increase, as does the relative percentage of apoE protein. The high-carbohydrate diet increases plasma levels of both apoE-rich (2-fold) and apoE-poor (3.5-fold) VLDL and plasma VLDL exhibited decreased immunoreactivity with a monoclonal antibody directed against epitopes located near the LDL receptor recognition region of apoB-100 (Keidar *et al.*, 1990). The dietary carbohydrate-induced changes in the distribution of VLDL subfractions and in the expression of apoB-100 epitopes would be expected to play a role in the metabolic channeling of VLDL, either toward direct catabolism or the delipidation cascade to LDL, and could in part account for the findings of *in vivo* VLDL kinetic studies of changes in VLDL fractional catabolic rates via these two pathways.

While the evidence demonstrates that an acute increase in dietary carbohydrate intake increases plasma triacylglycerol levels in most individuals, the question remains whether a more gradual transition to a low-fat, high-carbohydrate diet also results in carbohydrate-induced hypertriglyceridemia. Studies by Ullman *et al.* (1991) tested this possibility by gradually increasing the carbohydrate of test diets from 45 to 65% while reducing fat calories from 40 to 20% by incorporating 5% increments every 10 days. These investigators found that total, LDL, and HDL cholesterol and apoB and apoA-I levels decreased as fat calories decreased; however, there was no significant increase in total or VLDL triacylglycerol levels. The LDL:HDL ratio declined from 4.1 to 3.6 between the 40% ene and 20% ene fat diets. While these data suggest that a gradual adaptation to a high-carbohydrate diet does not increase plasma VLDL levels, it should be noted that not only was dietary fat quantity a variable but also that changes occurred in dietary fat quality with an increase in the P:S ratio from 0.4 (40% ene fat) to 1.6 (20% ene fat); therefore, it is uncertain what effects occurred because of changes in the amount of fat versus the type of fat (Jones *et al.*, 1987). It was also noted that the simple sugar content of the diets was held constant (Ullman *et al.*, 1991), which could also partially account for the lack of a hypertriglyceridemic response (Liu *et al.*, 1984).

C. FAT QUANTITY AND LDL LEVELS

Many studies have shown that a reduction in fat calories lowers plasma LDL cholesterol levels (Tables VI and VII) and *in vivo* studies of plasma LDL turnover

kinetics indicate that dietary fat quantity has a significant effect on the catabolism of VLDL to LDL, on the direct production of LDL, and on LDL fractional catabolic rates. Nestel *et al.* (1979) reported that when patients were shifted from a 40% ene to a 15% ene fat diet with the same P:S ratio and cholesterol content, LDL apoB fractional catabolic rates were increased from 0.24 to 0.32 pools/day. With a similar dietary pattern maintaining a dietary fat P:S ratio and the amount of cholesterol, Cortese *et al.* (1983) found that a reduction in dietary fat calories from 45 to 25% increased LDL apoB fractional catabolic rates from 0.26 to 0.30 pools/day and that LDL apoB production rates decreased from 9.6 to 7.7 mg/kg/day. Similar results were reported by Ginsberg *et al.* (1981), who found that when patients reduced their caloric intake of fat from 40% to zero, LDL apoB fractional catabolic rates fell.

Studies by Abbott *et al.* (1990) in Pima Indians fed high- and low-fat diets demonstrated that a dietary shift from 42% ene fat (P:S 0.29) to 21% ene fat (P:S 1.0) resulted in decreased LDL apoB production rates with no change in fractional catabolic rates. The decreased production rate of LDL apoB on the low-fat diet in part resulted from a decreased rate of conversion of VLDL to LDL coupled with an increased direct removal of VLDL (Abbott *et al.*, 1990). Why the increase in the P:S ratio of the dietary fat did not increase LDL apoB fractional catabolism is unclear, considering that the studies by Nestel *et al.* (1979) and Cortese *et al.* (1983) found an increased LDL fractional catabolic rate when dietary fat quantity was reduced and fat quality remained constant. Abbott *et al.* (1990) hypothesized that the low-fat diet resulted in a modest increase in LDL clearance, based on analysis of the relationship between diet-induced changes in LDL apoB production rates and in LDL apoB concentrations, and suggest that this effect could be due to an increased affinity of plasma LDL for its receptor during intake of the low-fat diet (Keidar *et al.*, 1989). Whether this carbohydrate-induced change in the interactions between LDL and their receptors accounts for the observed effects on LDL turnover, and how this might relate to LDL apoB fractional catabolic rates, remain to be determined; however, *in vitro* studies suggest that this is a real possibility.

Not only does a low-fat diet alter endogenous LDL metabolism, but, as noted above, there is evidence that low-fat, high-carbohydrate diets also have significant effects on LDL composition and receptor interactions. Studies by Keidar *et al.* (1989) have shown that when dietary fat calories are reduced from 40% ene to 1%, plasma levels of LDL and HDL cholesterol, apoA-I, and apoB are reduced, whereas VLDL cholesterol and triacylglycerol are increased. LDL triacylglycerol content increases from 3 to 4% of particle mass and there is an accumulation of smaller LDL particles in the plasma. Radioimmunoassays demonstrated that the high-carbohydrate, fat-free diet altered epitope expression of LDL apoB and *in vitro* studies of LDL association and degradation by human skin fibroblasts indicated that LDL obtained during the high-carbohydrate diet

period were more rapidly degraded than were basal LDL (Keidar *et al.*, 1989). These authors suggest that the observed changes in LDL composition, epitope expression, and *in vitro* metabolism associated with intake of a high-carbohydrate diet were the result of metabolic changes in intracellular lipoprotein assembly and/or postsecondary metabolism (Keidar *et al.*, 1989, 1990), which could be related to diet-induced changes in the conversion of VLDL to LDL (Abbott et al., 1990) and in LPL and HTGL activities (Brinton *et al.*, 1990). Studies in the guinea pig also indicate that as dietary fat calories are increased from 7.5 to 15% (by weight), LDL composition is altered and the density decreased even though the same dietary fats are fed (Fernandez and McNamara, 1991b). Effects of dietary fat quantity on lipoprotein size and composition, particularly on apoB epitope expression and receptor–ligand interactions, no doubt have major effects on lipoprotein metabolism.

D. FAT QUANTITY AND HDL LEVELS

Most studies indicate that reducing dietary fat calories lowers plasma HDL cholesterol levels and that drastic reductions in fat calories, with replacement by carbohydrates, significantly lower plasma levels of both HDL cholesterol and apoA-I. As discussed above, the available data indicate that an isocaloric exchange of PUFA for SFA, within the range of P:S ratios of 0.2 to 2.0, has only a modest effect on plasma HDL cholesterol levels. Based on these findings, it can be hypothesized that the plasma HDL cholesterol lowering observed when both the amount and the type of dietary fat are modified result from lowering fat quantity, not from changes in fat quality. Studies by Ehnholm *et al.* (1982, 1984), Brussaard *et al.* (1982), Kushi *et al.* (1985), Grundy *et al.* (1986, 1988; see Fig. 6), and Mensink *et al.* (1989) demonstrated that lowering dietary SFA calories from between 30 and 40% ene to 20% or lower reduced plasma total HDL cholesterol levels, with the reduction occurring in the HDL_2 fraction; the HDL_3 fraction was unaffected by the reduction in fat calories. Similar changes were observed whether the percentage of PUFA in the diet was constant (Ehnholm *et al.*, 1984; Kushi *et al.*, 1985; Mensink *et al.*, 1989), increased (Ehnholm *et al.*, 1982), or decreased (Brussaard *et al.*, 1982; Grundy *et al.*, 1986, 1988). Similar data have been reported by Wolf and Grundy (1983) from studies in men fed diets of the same P:S ratio but differing in fat quantity, where plasma HDL levels decreased 6% when fat calories were reduced from 40 to 30% ene. Studies comparing high-fat versus low-fat diets with the same P:S ratio in endurance athletes have also shown that the low-fat diet lowers plasma HDL cholesterol levels (Lukaski *et al.*, 1984).

There are, however, conflicting reports demonstrating that reductions in dietary fat calories do not lower plasma HDL levels (Liu *et al.*, 1983; Weisweiler *et al.*, 1985; Jones *et al.*, 1987; Baggio *et al.*, 1988; Iacono and Dougherty, 1991).

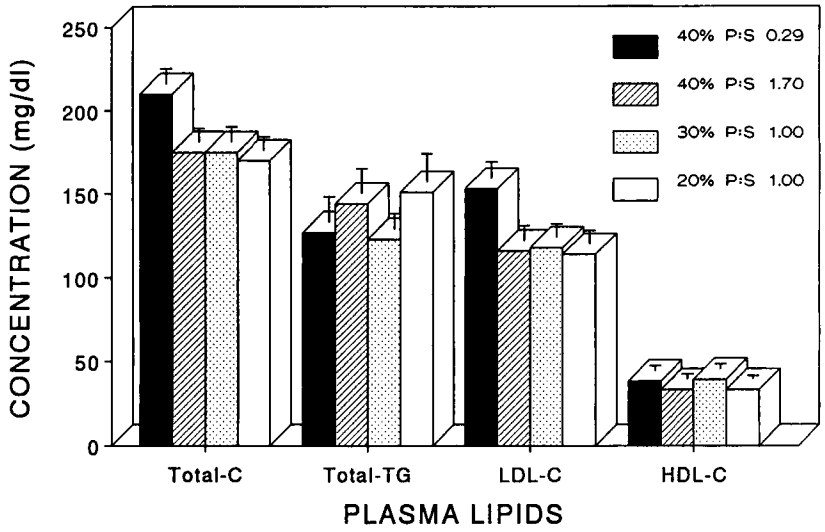

FIG. 6. Comparison of the effects of intake of 40% of calories as saturated fatty acids versus 40, 30, or 20% of calories as polyunsaturated fatty acids on plasma lipid and lipoprotein levels. Subjects (n = 9) were fed diets containing either 40% ene SFA (P:S 0.29), 40% ene PUFA (P:S 1.70), 30% ene PUFA (P:S 1.00), or 20% PUFA (P:S 1.00) for 4 weeks after which plasma lipids and lipoproteins were determined. Plasma total and LDL cholesterol levels were significantly lower ($P < 0.05$) on all three PUFA diets compared to the SFA phase; HDL cholesterol was significantly lower on the 20% ene PUFA diet. (After Grundy *et al.*, 1986.)

In part, the absence of a reduction in plasma total HDL cholesterol levels in response to initiation of a low-fat diet could be due to the fact that studies have shown that when reductions are observed, the reduction occurs in the HDL_2 subfraction (Mensink *et al.*, 1989; Ehnholm *et al.*, 1982, 1984; Brussaard *et al.*, 1982; Kushi *et al.*, 1985), and in some studies the changes in plasma total HDL are minor while changes in the HDL_2 subfraction are significant (Brussaard *et al.*, 1982; Fumeron *et al.*, 1991b). It is possible that the combinations of intraindividual variability in plasma HDL levels coupled with analysis of total HDL cholesterol rather than the HDL_2 subfraction, which represents less than half of the total HDL, obscured significant effects of changes in fat quantity.

Analysis of the effects of dietary fat quantity on plasma levels of apoA-I and apoA-II has resulted in variable results, with the majority of the data indicating that as dietary fat calories are reduced, plasma apoA-I levels decrease, and that there is little or no change in plasma apoA-II concentrations (Gonen *et al.*, 1981; Keidar *et al.*, 1989, 1990; Brinton *et al.*, 1990; Ullman *et al.*, 1991). In these studies the decrease in apoA-I levels appears to be proportional to the decrease in dietary fat calories over the entire range.

Zimmerman *et al.* (1986) investigated the effects of reducing dietary fat calories from 45% ene to 29% (P:S 1.0) on apoA-I kinetics in normolipidemic males. The results indicated that when dietary fat calories were lowered, plasma HDL cholesterol and apoA-I levels were slightly increased and apoA-II levels were unchanged. During intake of the low-fat diet there were significant increases in both the fractional catabolic rate (+6%) and the production rate (+10%) of HDL apoA-I. Similar data for HDL apoA-I kinetics were observed in a group of hypertriglyceridemic patients tested with similar diets. Analysis of HDL composition indicated effects of dietary fat quantity on HDL composition and subclass distribution, leading the authors to conclude that moderate changes in dietary fat content affect HDL levels, composition, and apoA-I metabolism (Zimmerman *et al.*, 1986).

Extreme changes in dietary fat quantity, e.g., 40% ene fat to less than 10% ene, result in striking decreases in plasma HDL levels (Blum *et al.*, 1977; Schaefer *et al.*, 1981; Snook *et al.*, 1985; Brinton *et al.*, 1990). Blum *et al.* (1977) conducted studies in four female patients and found that reducing dietary fat calories from 40% ene to zero resulted in a 36% reduction in plasma HDL cholesterol levels and a 35% increase in HDL fractional catabolic rates; HDL production rates were unaffected by the removal of dietary fat. Analysis of apoA-I and apoA-II kinetics in 13 patients fed 42% ene fat (S:M:P = 24:16:2) versus 8% fat (S:M:P = 2:4:2) demonstrated significant reductions in plasma levels of HDL cholesterol (−29%) and apoA-I (−23%) in subjects on the low-fat diet; apoA-II levels were unaffected. The lowering of plasma apoA-I levels in subjects on the low-fat diet was associated with an increased apoA-I fractional catabolic rate (+11%) and a 14% decrease in apoA-I production rate (Brinton *et al.*, 1990). The fractional catabolic rate of apoA-II was increased by 6% in subjects on the low-fat diet, but the production rate was unchanged. The authors concluded that the major effect of a low-fat diet on plasma apoA-I metabolism resulted from changes in the production rate; the data also indicated that intraindividual variance in plasma apoA-I levels was due to differences in the fractional catabolic rates (Brinton *et al.*, 1990).

The decrease in plasma HDL cholesterol levels brought about by a reduction in dietary fat calories is also apparent from epidemiological studies. Knuiman *et al.* (1987) reviewed the relationship between dietary fat calories and plasma HDL levels and found a significant negative relationship between carbohydrate intake and HDL cholesterol concentrations. The epidemiology data indicated that for every 10% ene increase in carbohydrate calories, there was a 3 mg/dl decrease in HDL cholesterol level, a value consistent with findings from clinical diet trials of a decrease of 4 mg/dl in HDL for every 10% ene exchange of carbohydrates for fats (Knuiman *et al.*, 1987).

Lowering dietary fat calories clearly has an effect on plasma HDL cholesterol and apolipoprotein levels in humans; however, it is probable that the extent of

the lowering of HDL relates to a number of factors, including the type of carbohydrate used to replace fat calories (Liu *et al.*, 1984), the degree of endogenous hypertriglyceridemia, the extent of plasma triacylglycerol increase on the low-fat diet, and the P:S ratio of the low-fat diet (Jones *et al.*, 1987). From both clinical and epidemiological studies it can be estimated that for every 10% exchange of fat for carbohydrate calories, plasma HDL cholesterol levels will decrease 3–4 mg/dl.

E. FAT QUALITY, INTRAVASCULAR LIPOPROTEIN PROCESSING, AND RECEPTOR-MEDIATED LIPOPROTEIN CATABOLISM

Relatively little is known regarding the effects of dietary fat quantity on the enzymes and receptors involved in the intravascular processing of plasma lipoproteins in humans. Such information is essential in order to determine the effects of fat calories on the metabolism of the plasma lipoproteins, and might explain some of the changes in VLDL, LDL, and HDL kinetics resulting from reductions in fat calories.

1. LPL/HTGL

Reducing dietary fat from 42 to 9% ene (with an increase in the P:S ratio and reduction in dietary cholesterol) results in significant reductions in plasma postheparin lipase activity, both LPL and HTGL, in humans (Brinton *et al.*, 1990). If such reductions occur with modest reductions in dietary fat, this could partially explain the decreased VLDL triacylglycerol catabolism observed in some studies and the increased direct removal of plasma VLDL with reduced conversion to IDLs and LDL (Ginsberg *et al.*, 1981; Abbott *et al.*, 1990). The higher LPL and HTGL levels during consumption of a high-fat versus a low-fat diet have also been observed in rats (Coiffier *et al.*, 1987).

2. LDL Receptor Expression

Studies have shown that when guinea pigs are shifted from a 19 to a 35% ene fat diet (either corn oil, olive oil, or lard), hepatic LDL receptor levels do not differ within dietary fat groups, and that the increased expression of apoB/E receptors in animals on the PUFA corn oil-based diet is evident at both levels of dietary fat. There is, however, an increase in the affinity of the receptor for LDL, and this increase in the affinity of the receptor for LDL, and this increase was observed in all three dietary fat groups (Fernandez and McNamara, 1991a). Studies in humans have shown that a low-fat diet results in a smaller LDL particle having an increased rate of degradation when tested *in vitro* (Keidar *et al.*, 1989). *In vivo* studies of LDL kinetics in patients on high- and low-fat diets have not

investigated rates of receptor-mediated LDL catabolism specifically, and the relevance of the animal model findings and the *in vitro* studies to *in vivo* metabolism remains speculative.

Effects of dietary fat quantity on plasma LCAT and CETP activity and on hepatic HDL receptor levels are unknown at this time. Considering the plasma HDL-lowering effects of a low-fat, high-carbohydrate diet, it is important that the effects of this dietary change on parameters of HDL metabolism be defined in more detail in order to better understand the metabolic consequences of dietary fat reductions.

VIII. PLASMA LIPID/LIPOPROTEIN RESPONSES TO *n*-3 POLYUNSATURATED FATTY ACIDS

In the 1970s, Bang *et al.* (1976) and Dyerberg *et al.* (1975) hypothesized that the low incidence of CVD in Greenland Eskimos was due to increased intake of marine oils, consisting of long-chain, ω-3 (*n*-3) PUFA, primarily eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Similar findings have been observed from studies of Japanese fisherman and farmers (Hirai *et al.*, 1989) and in epidemiological studies in The Netherlands (Kromhout *et al.*, 1985) and in Chicago (Shekelle *et al.*, 1985). It should be noted that in two studies, the Honolulu Heart Study (Curb and Reed, 1985) and a Norwegian study (Vollset *et al.*, 1985), no effect of fish intake on CHD incidence was found. In studies wherein significant effects of fish intake on CHD incidence have been reported, intake of fish in the diet provided between a low of 0.5 and a high of 7 g/day of *n*-3 PUFA, levels substantially less than those usually used in clinical studies of the effects of fish oils on plasma lipid and lipoprotein levels and lipoprotein metabolism.

Numerous metabolic studies have demonstrated that intake of *n*-3 PUFA has a significant hypolipidemic effect, with the major response being a reduction in plasma triacylglycerol levels (Herold and Kinsella, 1986; Harris, 1989; Nestel, 1990; Kinsella *et al.*, 1990). In addition, studies have shown that intake of *n*-3 PUFA result in a number of changes in eicosanoid metabolism, which affect platelet and endothelial cell interactions in the vessel wall and macrophage metabolism (Leaf and Weber, 1988; Weber and Leaf, 1991; Kinsella *et al.*, 1990). These changes result in a decreased thrombic tendency, which could retard the initiation and progression of atheromatous lesions (Kinsella *et al.*, 1990). The combined antithrombic and plasma lipid-lowering effects of dietary *n*-3 PUFA are thought to account for the reduced CHD incidence in populations consuming diets high in fish oils.

There have been a number of recent reviews that provide extensive coverage of the hypolipidemic and antiatherogenic effects of *n*-3 PUFA (Harris, 1989;

Leaf and Weber, 1988; Kinsella *et al.*, 1990; Nestel, 1990; Weber and Leaf, 1991). The reader is referred to these reviews for details of early studies of the effects of *n*-3 PUFA on plasma lipids and lipoproteins, and for discussions of the effects of *n*-3 PUFA on eicosanoid synthesis and regulation and other non-lipoprotein-mediated effects of *n*-3 PUFA on atherogenesis and thrombosis, which are covered in this review.

One of the complicating factors in comparing results of studies of the effects of *n*-3 PUFA on lipoprotein levels and metabolism is the type of dietary fat used as the control for any one study, whether SFA or *n*-6 PUFA. If *n*-3 PUFA have singularly unique effects on plasma lipoprotein levels due to their C20–C22 chain length, number of double bonds, and *n*-3 structure (as the data seem to indicate), then their effects could in theory be compared to either SFA or *n*-6 PUFA. On the other hand, if their effects are the result of a combination of its polyunsaturation, *n*-3 structure, and chain length, then testing plasma lipid responses and metabolic changes relative to *n*-6 PUFA would be more appropriate and informative. The literature contains some studies wherein diets containing *n*-3 PUFA are compared to SFA and other studies wherein the comparison is to *n*-6 PUFA; in few cases are they compared to both. It is rarely recognized that conflicting study results are in part due not so much to differences in response to *n*-3 PUFA as to differences at the start of the study due to the saturation of the baseline dietary fat. It would be expected that the effects of *n*-3 PUFA on lipoprotein metabolism would be different if in one study the comparison diet contains lard, with its SFA effects, and in another the comparison diet contains safflower oil, which has its own *n*-6 PUFA effects on lipoprotein metabolism. In addition, the effects of EPA and DHA on lipoprotein metabolism have been shown to differ, and the use of various "fish oils" containing different ratios of EPA to DHA adds yet another variable. For example, it has been reported that EPA effectively lowers plasma triacylglycerol levels and DHA effectively lowers plasma cholesterol levels in rats (Kobatake *et al.*, 1984). Such differences in effects of specific *n*-3 PUFA have contributed to some of the confusion regarding effects of the general classification of *n*-3 PUFA on lipoprotein levels and metabolism.

With these considerations in mind, the composite data clearly demonstrate significant effects of *n*-3 fatty acids on plasma VLDL levels in normolipidemic, hypercholesterolemic, hypertriglyceridemic, and combined hyperlipidemic patients (Harris, 1989). An analysis of all available data on the effects of *n*-3 fatty acids on plasma lipid and lipoprotein levels has been reported by Harris (1989), and demonstrates that the primary effects of *n*-3 PUFA intake are a reduction in plasma triacylglycerol levels, with either no change or modest increases in plasma LDL and HDL levels (Table VIII). As shown in Fig. 7, there was a clear dose–response relationship between changes in plasma triacylglycerol levels and the dose of *n*-3 fatty acids administered. It is also clear from the data presented in Table VIII and Fig. 7 that the relative decrease in plasma triacylglycerol levels

TABLE VIII

PLASMA LIPID AND LIPOPROTEIN RESPONSES TO INTAKE OF n-3 POLYUNSATURATED FATTY ACIDS IN PATIENTS WITH VARIOUS FORMS OF HYPERLIPIDEMIA[a]

Patient classification	n	Change in plasma lipids/lipoproteins			
		Total cholesterol	LDL cholesterol	HDL cholesterol	Triacyl-glycerol
Normolipidemic	596	−1.8%	+0.03%	+3.4%	−25.2%
Hyper cholesterolemic	37	+1.3%	+2.3%	+3.8%	−20.3%
Combined hyperlipidemic	194	−2.0%	+5.6%	+7.3%	−38.0%
Hyper triglyceridemic	101	−7.8%	+29.9%	+9.7%	−52.2%

[a]Data from individual studies as compiled and analyzed by Harris (1989) and references therein.

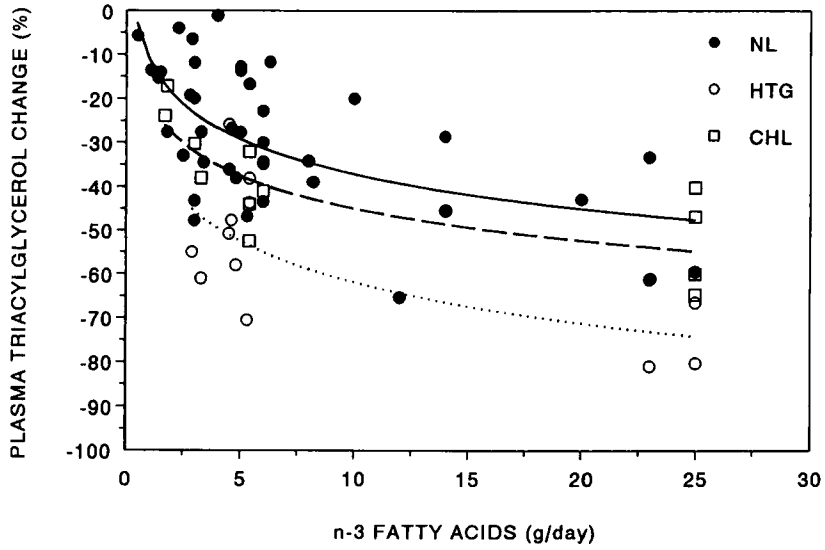

FIG. 7. Relationship between dose of n-3 fatty acids and response of plasma triacylglycerol in normolipidemic (NL), hypertriglyceridemic (HTG), and combined hyperlipidemic (CHL) patients. Composite data relating percent changes in plasma triacylglycerol to intake of n-3 fatty acids (mg/day) in normolipidemic (●), hypertriglyceridemic (○), and combined hyperlipidemic (□) patients. (After Harris, 1989, and references therein.)

in response to *n*-3 fatty acid intake is positively related to the extent of hypertriglyceridemia exhibited by the study subjects (Harris, 1989).

A. EFFECTS OF *n*-3 POLYUNSATURATED FATTY ACIDS ON CHYLOMICRON METABOLISM

Studies of postprandial triglyceridemia in humans consuming *n*-3 PUFA have demonstrated a significant lowering of postprandial total and chylomicron triacylglycerol concentrations and an improved lipemic response (Harris and Connor, 1980; Harris *et al.*, 1988; Saynor *et al.*, 1984; Weintraub *et al.*, 1988; Brown and Roberts, 1991). Peak postprandial triacylglycerol levels fall between 35 and 50%, depending upon the dosage of fish oil in the test diets. Harris *et al.* (1988) demonstrated that the response was dependent upon chronic intake of fish oil and that when fish oils were tested in the acute phase of the postprandial challenge, there was no reduction in postprandial lipemia unless fish oils had been the chronic diet. Similar results have been reported by Weintraub *et al.* (1988). On the other hand, it did not matter whether the acute diet for the postprandial test contained SFA or *n*-3 PUFA; the reduced lipidemic response persisted in the fish oil treatment group (Harris *et al.*, 1988; Weintraub *et al.*, 1988). The data suggest that intake of *n*-3 PUFA reduces the synthesis and/or secretion of chylomicrons from the enterocyte. Studies by Brown and Roberts (1991) of the effects of moderate fish oil intake (5 g/day) on lipemic responses indicated that both the control group (5 g/day olive oil) and the fish oil group had similar postprandial increases in chylomicron retinyl esters (a marker for chylomicrons and chylomicron remnant metabolism) during the first 2 hr, but that the postprandial response of the fish oil group was significantly lower after this time, with a lower peak value. Such an observation is consistent with decreased chylomicron synthesis and secretion.

It has also been suggested that intake of *n*-3 PUFA could accelerate clearance of postprandial chylomicrons and their remnants due to the reduction in plasma VLDL levels (Harris *et al.*, 1988). This hypothesis is based on the concept that since chylomicrons and VLDL compete for the same lipolytic mechanisms via LPL, the clearance of chylomicrons will be accelerated when VLDL levels are reduced. Since most studies suggest that postheparin LPL and HTGL activities are not stimulated by intake of *n*-3 PUFA (see below), this hypothesis could account for changes in chylomicron clearance, if differences in clearance do in fact exist. To date there are no data on the effects of *n*-3 polyunsaturated fatty acids on chylomicron clearance rates in humans or in animal models. *In vitro* studies have demonstrated that both *n*-6 and *n*-3 PUFA-containing chylomicrons are hydrolyzed by purified LPL faster than are SFA-containing particles (Weintraub *et al.*, 1988).

The most reasonable explanation for the reduction in postprandial triacylglycerol levels in patients fed for a period of time with *n*-3 PUFA is that the *n*-3 fatty acids inhibit triacylglycerol synthesis, similar to what has been shown in the liver (see below), and that this response, in combination with a reduction in VLDL levels, results in a modest increase in intravascular chylomicron catabolism due to decreased competition for the lipolytic enzymes. While this is an appealing hypothesis, studies by Chautan *et al.* (1991) in rats fed *n*-3 PUFA found increased activities of intestinal enzymes involved in cholesteryl ester, triacylglycerol, and phospholipid synthesis. The decreased postprandial increase in plasma chylomicrons due to *n*-3 PUFA intake would appear to involve as-yet undefined mechanisms.

B. EFFECTS OF *n*-3 POLYUNSATURATED FATTY ACIDS ON VLDL METABOLISM

Studies in animal model systems and in humans have uniformily demonstrated the plasma triacylglycerol-lowering effect of *n*-3 PUFA (Nestel, 1990). In virtually every study reported, intake of EPA and DHA has been shown to significantly lower plasma total and VLDL triacylglycerol levels in rats, rabbits, swine, a variety of nonhuman primates, and humans, and the extent of lowering is positively related to the extent of the hypertriglyceridemia during the baseline diet phase (Simons *et al.*, 1985). *In vitro* and *in vivo* studies have shown that intake of *n*-3 PUFA lowers hepatic triacylglycerol synthesis and secretion rates. Analysis of triacylglycerol synthesis and secretion by perfused livers of rats fed 15% (by weight) safflower oil or fish oil indicated that intake of the *n*-3 PUFA reduced lipogenesis and triacylglycerol secretion with a corresponding increase in fatty acid oxidation (Wong *et al.*, 1984). Studies of triacylglycerol synthesis and secretion by isolated hepatocytes of rats and rabbits fed *n*-3 PUFA have shown decreased VLDL triacylglycerol synthesis and secretion rates compared to animals fed either SFA- or *n*-6 PUFA-rich diets (Wong *et al.*, 1985; Nossen *et al.*, 1986; Rustan *et al.*, 1988; Benner *et al.*, 1990). *In vitro* studies have shown that, whereas oleate stimulates triacylglycerol synthesis and secretion by isolated hepatocytes, EPA has an inhibitory effect (Nossen *et al.*, 1986; Rustan *et al.*, 1988). Rustan *et al.* (1988) have shown that addition of EPA to isolated rat hepatocytes results in inhibition of acyl-CoA:1,2-diacylglycerol acyltransferase, the last step in triacylglycerol synthesis, which in part explains the observation that addition of EPA to hepatocytes inhibits the stimulatory effect of oleate on triacylglycerol synthesis and secretion (Nossen *et al.*, 1986; Rustan *et al.*, 1988).

It should be noted, however, that not all studies have detected reduced activity of diacylglycerol acyltransferase in response to intake of *n*-3 PUFA (Marsh *et al.*, 1987; Al-Shurbaji *et al.*, 1991). The evidence indicates that EPA inhibits

triacylglycerol synthesis while being preferentially incorporated into phospholipids (Wong *et al.*, 1985; Rustan *et al.*, 1988; Benner *et al.*, 1990; Ribeiro *et al.*, 1991). In addition, intake of *n*-3 fatty acids has been reported to increase fatty acid oxidation (Wong *et al.*, 1984; Yamazaki *et al.*, 1987; Ribeiro *et al.*, 1991), increase phospholipid synthesis (Rustan *et al.*, 1988; Benner *et al.*, 1990), and decrease hepatic phosphatidate phosphohydrolase (Marsh *et al.*, 1987; Wong and Marsh, 1988; Al-Shurbaji *et al.*, 1991), all of which would result in significant alterations in the availability of triacylglycerols for VLDL production.

While the inhibitory effects of *n*-3 PUFA on triacylglycerol synthesis and secretion are consistently found, the effects on VLDL apoB production are more variable. Wong and Nestel (1987) reported that secretion of VLDL apoB by HepG2 cells was reduced by incubation with EPA as compared to oleate, and similar findings have been reported from rat liver perfusion studies comparing oleate versus *n*-3 PUFA in the perfusate (Wong and Marsh, 1988). Subsequent studies in HepG2 cells demonstrated that incubation with EPA or DHA inhibited apoB synthesis but had no effect on apoB mRNA levels (Wong *et al.*, 1989). In contrast to the data from acute studies, liver perfusion studies of African green monkeys fed high-cholesterol diets containing either lard or fish oil indicated decreased VLDL triacylglycerol and cholesteryl ester secretion but no change in VLDL apoB secretion in the fish oil-fed animals (Parks *et al.*, 1989a). Similar data have been obtained from studies of lipoprotein metabolism in isolated hepatocytes of rats fed either fish oil or corn oil and indicated that *n*-3 PUFA decreased apoB synthesis at a posttranscriptional level, since apoB mRNA levels were unaffected, with a large proportion of newly synthesized apoB being degraded intracellularly (Ribeiro *et al.*, 1991). It would appear from these data that there is a significant difference between the chronic and acute effects of *n*-3 PUFA on apoB production.

Most studies of the effects of *n*-3 PUFA on VLDL synthesis have used a mixture of *n*-3 fatty acids, yet it would appear that not all *n*-3 fatty acids have the same effect. Studies by Kestin *et al.* (1990) have shown that *n*-3 fish oils high in EPA and DHA lower plasma VLDL levels in humans, whereas *n*-3 linolenic acid was not as effective. Studies in HepG2 cells have shown that although both EPA and DHA inhibit apoB synthesis, EPA inhibits triacylglycerol secretion, with no effect on synthesis (Wong *et al.*, 1989). Similar data have been obtained in clinical studies of fish oils with varying proportions of EPA and DHA, which indicate that EPA lowers plasma triacylglycerol levels to a greater extent than cholesterol levels whereas DHA lowers triacylglycerol and cholesterol levels equally (Childs *et al.*, 1990). What these studies suggest is that not all *n*-3 PUFA have the same effects on lipid and lipoprotein metabolism and that EPA and DHA may have unique and independent effects on VLDL synthesis and secretion *in vivo*.

Intake of *n*-3 PUFA not only alters VLDL secretion but also alters VLDL composition and *in vivo* catabolism. Studies in humans have shown that intake of *n*-3 PUFA results in smaller VLDL particles (Harris *et al.*, 1990b; Inagaki and Harris, 1990), and similar findings have been reported for African green monkeys fed *n*-3 PUFA (Parks *et al.*, 1989a). These alterations in VLDL composition would be predicted to affect intravascular VLDL metabolism and the metabolic channeling of VLDL to either LDL or direct removal.

In vivo studies of VLDL kinetics demonstrate that intake of *n*-3 PUFA decreases VLDL production rates, increases fractional catabolic rates, and modifies the metabolic channeling of VLDL apoB. Studies in roosters fed 10% (by weight) corn oil or fish oil indicate that the fish oil diet decreased rates of VLDL cholesterol and triacylglycerol secretion (Daggy *et al.*, 1987). When 0.5% cholesterol was added to the test diets and VLDL kinetic parameters determined, the data indicated that intake of the fish oil diet decreased VLDL cholesterol production rates, increased the fractional removal rate of VLDL cholesterol fivefold, and lowered the mass flux of VLDL to LDL cholesterol by 60% (Daggy *et al.*, 1987). Analysis of VLDL apoB kinetics in swine fed 9% (by weight) corn oil or fish oil demonstrated that intake of the fish oil diet lowered VLDL apoB pool size and increased the fractional catabolic rate and the rate of VLDL flux to LDL, with a reciprocal decrease in VLDL flux by direct removal; VLDL apoB production rates were unaffected by intake of the fish oil diet (Huff and Telford, 1989). As discussed below, there is a significant effect of fish oil intake on LDL apoB metabolism, including a reduction in the direct production of LDL apoB. Why the intake of the fish oil diet does not lower VLDL apoB production in swine is unclear, but could relate to the very low VLDL apoB levels in both dietary groups, 0.6 mg/dl in the corn oil group and 0.4 mg/dl in the fish oil group. It is possible that the low level attained during intake of the corn oil diet could not be further reduced by intake of fish oil (Huff and Telford, 1989).

It is possible that the lack of an *n*-3 PUFA effect on VLDL apoB production rates in swine relates to specificity toward reductions in triacylglycerol synthesis and secretion with minimal changes in apoB secretion. Such a hypothesis is consistent with liver perfusion studies in African green monkeys fed high-cholesterol diets containing either lard or fish oil (Parks *et al.*, 1989a). In studies of hepatocytes from rats fed corn oil versus fish oil, intake of *n*-3 PUFA has been shown to decrease apoB synthesis at a posttranscriptional level (Ribeiro *et al.*, 1991). Studies of *n*-3 PUFA effects on VLDL production by the human hepatoma cell line HepG2 cells in culture are complicated by differences in acute versus chronic effects. The evidence suggests that *n*-3 PUFA intake reduces apoB synthesis as compared to *n*-6 PUFA intake, but has no significant effect when compared to SFA.

Studies of the effects of *n*-3 PUFA on VLDL synthesis in African green

monkeys are difficult to interpret for two reasons: first, this nonhuman primate has extremely low levels of VLDL, and second, the baseline diets contain 42% ene lard and 700–800 mg cholesterol/1000 kcal and are compared to a fish oil diet with the same fat calories and dietary cholesterol (Parks *et al.*, 1987, 1989a,b, 1990a,b). Under such conditions it is difficult to determine if the magnitude of the hypercholesterolemia induced by the high-cholesterol intake overwhelms any subtle effects of the *n*-3 PUFA. It is also unclear whether any observed effects result from addition of the *n*-3 PUFA or removal of the saturated fat. Liver perfusion studies in this animal model have shown that intake of *n*-3 PUFA lowers the rates of accumulation of triacylglycerol and cholesterol but not apoB and that the secreted VLDL are smaller, with triacylglycerol:protein and cholesterol:protein ratios reduced by as much as 50% (Parks *et al.*, 1989a).

A number of studies on the effects of *n*-3 PUFA on VLDL metabolism, both triacylglycerol and apoB, have been carried out in normolipidemic and hyperlipidemic humans. These studies demonstrate that the hypotriglyceridemic effect of *n*-3 fatty acids results from a decreased VLDL triacylglycerol production rate and small increases in the fractional catabolic rate (Nestel *et al.*, 1984; Sanders *et al.*, 1985; Harris *et al.*, 1990b). Analysis of VLDL apoB kinetics indicates that fish oil intake decreases the production rate and increases the fractional catabolic rate in normolipidemic subjects. The response of hypertriglyceridemics is similar in that fish oil intake lowers VLDL apoB production but has variable effects on the fractional catabolic rate (Nestel *et al.*, 1984).

From studies in animal model systems and in humans it is clear that intake of *n*-3 PUFA lowers VLDL triacylglycerol production, resulting in a significant hypotriglyceridemic effect in both normolipidemic and hypertriglyceridemic individuals. This response is seen even when patients are challenged with a high-carbohydrate diet in that intake of *n*-3 fatty acids prevents the carbohydrate-induced hypertriglyceridemia (Harris *et al.*, 1984). The ability of *n*-3 fatty acids to inhibit VLDL triacylglycerol production appears to be equal for EPA and DHA in humans (Harris, 1989), and its efficacy in reducing elevated plasma triacylglycerol levels in hypertriglyceridemic patients no doubt relates to the extent of VLDL triacylglycerol overproduction, which is a characteristic of these individuals. It has also been suggested that since intake of *n*-3 fatty acids results in production of smaller, dense triacylglycerol-poor VLDL particles, these VLDL particles may be catabolized at a faster rate (Harris, 1989).

C. EFFECTS OF *n*-3 POLYUNSATURATED FATTY ACIDS ON LDL METABOLISM

The plasma LDL response to intake of *n*-3 PUFA is highly variable, which in large part is due to the fat composition of the baseline diet, the amount of

dietary cholesterol, and the hyperlipidemia exhibited by the study subjects. Animal model studies are complicated by the fact that animal species usually being studied have relatively low levels of plasma LDL to begin with. The data from most animal model studies indicate that *n*-3 PUFA have little if any lowering effect on plasma LDL cholesterol levels. Studies in monkeys fed high-cholesterol diets with either lard or fish oil indicate that intake of *n*-3 PUFA results in smaller LDL particles, which are thought to be derived from cholesteryl ester-poor VLDL (Parks and Bullock, 1987; Parks *et al.*, 1987, 1989a). Studies of *in vivo* LDL metabolism in swine fed diets containing either corn oil or fish oil have shown that *n*-3 PUFA intake decreases the LDL pool size, the direct production of LDL, and the fractional catabolic rate (Huff and Telford, 1989), whereas studies in rats have presented evidence that fish oil, as compared to coconut oil or safflower oil, results in decreased LDL production and increased receptor-mediated hepatic clearance of LDL (Ventura *et al.*, 1989). Analyses of LDL kinetics in roosters fed 10% (by weight) corn oil or fish oil demonstrate a decreased LDL fractional catabolic rate associated with a decreased production of LDL from VLDL (Daggy *et al.*, 1987). The variations in effects of *n*-3 PUFA on LDL metabolism in animal models illustrate the problems of having defined mechanisms regarding effects of *n*-3 fatty acids on LDL synthesis and catabolism.

Harris (1989) reported that analysis of data from numerous studies indicated that intake of *n*-3 fatty acids significantly increased plasma LDL levels in hypertriglyceridemic patients (Table VIII). Recent studies have reported similar findings (Dart *et al.*, 1989; Harris *et al.*, 1990a; Radack *et al.*, 1990; Kestin *et al.*, 1990; Reis *et al.*, 1990; Hughes *et al.*, 1990; Fumeron *et al.*, 1991a). Studies by Kestin *et al.* (1990) indicate that the increase in plasma LDL in response to fish oil intake occurs with *n*-3 PUFA of marine origin and does not occur with α-linolenic acid. In a similar manner, Childs *et al.* (1990) have shown that EPA intake increases LDL levels whereas DHA intake does not. While increased LDL apoB levels in response to *n*-3 PUFA intake have been reported in many studies, not all reports support this finding (Wilt *et al.*, 1989; Deck and Radack, 1989; Subbaiah *et al.*, 1989) and there is one report suggesting that the increase in LDL apoB occurs in males but not females (Dart *et al.*, 1989). Harris *et al.* (1990a) reported that plasma LDL levels increased in a dose–response manner as *n*-3 PUFA intake was increased, indicating that the observed responses of plasma LDL to fish oils vary with dosage and no doubt also with the composition of the baseline diet if *n*-3 PUFA are administered as a supplement.

In vivo LDL kinetic studies in patients fed varying levels of *n*-3 PUFA indicate a decrease in LDL pool size and production rates, with no significant effect on the fractional catabolic rate (Illingworth *et al.*, 1984). Interpretations of these data are complicated by the fact that both the type and the amount of PUFA in the diet were varied and the P:S ratio was changed from 0.5 to 1.2 when the

n-3 PUFA diet was fed. What the relative effects of changes in the P:S ratio versus addition of *n*-3 PUFA were on LDL kinetics cannot be distinguished. How decreased VLDL production results in elevated LDL levels is an important scientific question, and more information is needed to determine whether the effects of *n*-3 PUFA on LDL metabolism are mediated via changes in LDL production, either from VLDL or via direct production, and/or changes in LDL catabolism (Harris, 1989).

D. EFFECTS OF *n*-3 POLYUNSATURATED FATTY ACIDS ON HDL METABOLISM

There is a significant relationship between the metabolism of VLDL and HDL, and as such it would be predicted that since *n*-3 PUFA have such a significant effect on VLDL synthesis and catabolism that plasma HDL levels would also be affected by fish oil intake due to the decreased production of VLDL surface components, which are transferred to HDL during VLDL catabolism. In addition, *n*-3 PUFA-mediated reductions in plasma LCAT and CETP activities resulting from changes in phospholipid and cholesteryl ester fatty acids would also be expected to affect HDL levels. What is found is just the opposite in that intake of *n*-3 PUFA has been found to increase plasma HDL levels in most studies (Table VIII), and major decreases are only noted when extremely high doses of *n*-3 fatty acids are fed (Harris, 1989). The increase in plasma HDL usually is found in the HDL_2 fraction (Harris *et al.*, 1988; Harris, 1989; Lindgren *et al.*, 1991; Fumeron *et al.*, 1991a; Cobiac *et al.*, 1991), with an increase in the HDL_2:HDL_3 ratio (Abbey *et al.*, 1990a). Most studies find a small decrease in apoA-I levels during intake of *n*-3 PUFA (Lindgren *et al.*, 1991; Friday *et al.*, 1991; Cobiac *et al.*, 1991), but this usually is dependent upon the comparison diet, whether *n*-6 PUFA or SFA, and on the dosage. One interesting observation is that while intake of *n*-3 PUFA is able to block carbohydrate-induced hypertriglyceridemia, it does not block the reduction in plasma HDL and apoA-I levels usually found with low-fat, high-carbohydrate diets (Harris *et al.*, 1984).

Studies of apoA-I gene expression in hepatocytes of rats fed either 5% (by weight) corn oil or salmon oil indicated that intake of *n*-3 fatty acids had no effect on apoA-I mRNA levels, yet resulted in decreased rates of apoA-I synthesis and secretion (Ribeiro *et al.*, 1991). The data suggest that dietary *n*-3 PUFA lower apoA-I synthesis at a posttranscriptional level and that the decreased apoA-I synthesis in part accounts for the decreased plasma apoA-I concentrations (Ribeiro *et al.*, 1991). These data present a very different response of rat plasma apoA-I and HDL levels as compared to the data from human studies.

E. EFFECTS OF *n*-3 POLYUNSATURATED FATTY ACIDS ON INTRAVASCULAR LIPOPROTEIN METABOLISM AND LIPOPROTEIN RECEPTORS

1. LPL

Hartog *et al.* (1987) reported that postheparin lipase activities were similar in swine fed either 9% (by weight) mackerel oil or lard. In contrast, Groot *et al.* (1988) reported that postheparin LPL activity was decreased by 31% in swine fed 21% ene mackerel oil compared to lard. To further complicate matters, studies in rats indicated that intake of diets containing 42% ene coconut oil resulted in higher LPL activities compared to 42% ene diets containing fish oil (Haug and Hostmark, 1987). In studies comparing intake of 10% (by weight) corn oil, beef tallow, or fish oil on LPL activity, adipose tissue LPL was unaffected whereas muscle LPL activity was highest on the fish oil diet and lowest on the corn oil diet (Herzberg and Rogerson, 1989). In rats fed diets containing corn oil, lard, or fish oil, Baltzell *et al.* (1991) found no effects of dietary fat on adipose tissue LPL activity, whereas muscle LPL activity was increased during the *n*-3 PUFA diet, confirming the results of Herzberg and Rogerson (1989). In contrast to the data from animal studies, studies in humans indicate that intake of *n*-3 PUFA has no effect on postheparin LPL activity (Weintraub *et al.*, 1988; Harris *et al.*, 1988). What the human studies cannot determine is whether there are any specific tissue LPL effects (adipose versus muscle), as found in studies of rats on *n*-3 PUFA diets. The data would suggest that if *n*-3 PUFA have any effect on human LPL activity, it is probably small.

2. HTGL

Studies in rats indicate that intake of *n*-3 PUFA has no unique effects on HTGL activity. In rats, HTGL activity is decreased in animals fed *n*-3 PUFA compared to SFA (Haug and Hostmark, 1987; Baltzell *et al.*, 1991), but not compared to *n*-6 PUFA (Baltzell *et al.*, 1991). Defining effects of *n*-3 fish oils on HTGL activity obviously depends on the comparison diet, and since *n*-6 PUFA increase activity, there is no specific *n*-3 PUFA effect. Human studies have also demonstrated that intake of *n*-3 PUFA has no effect on postheparin HTGL activity (Weintraub *et al.*, 1988; Harris *et al.*, 1988).

3. LCAT

In baboons fed high-cholesterol diets containing either 11% (by weight) lard or fish oil, intake of the *n*-3 PUFA had no effect on plasma LCAT activity or

mass (Parks *et al.*, 1989a); however, studies indicated that LCAT activity was reduced due to incorporation of *n*-3 fatty acids into the sn-2 position of phospholipids. Based on these data, it is unclear whether the observed reduction in plasma LCAT activity in humans fed diets containing *n*-3 PUFA (Singer *et al.*, 1983; Abbey *et al.*, 1990a) resulted from a change in enzyme levels, activity, or specific effects of alterations in phospholipid composition, since the assays were carried out using the patients' plasma. The data suggest that observed changes in LCAT activity in response to intake of *n*-3 PUFA result from changes in enzyme–substrate interactions and not changes in enzyme activity.

4. CETP

Studies by Abbey *et al.* (1990b) in marmosets fed a standard colony diet with 10% (by weight) SFA–0.2% cholesterol, with or without addition of 0.8% EPA, indicated that EPA intake had no effect on plasma CETP activity when measured in the $d > 1.21$ lipoprotein-free fraction under standard conditions. When assayed in whole plasma, CETP activity in the marmosets was reduced by intake of *n*-3 fish oil, suggesting that any observed decrease in cholesteryl ester transfer relates to differences in the fatty acid moiety of the cholesteryl ester and not CETP levels. Studies in humans have yielded similar results (Abbey *et al.*, 1990a) in that addition of EPA to the diet results in decreased CETP activity measured in plasma but not when measured in the lipoprotein-free plasma fraction. The data also indicates that the reduction in plasma CETP activity occurred with EPA but not with α-linolenic acid (Abbey *et al.*, 1990a). As noted above regarding effects of *n*-3 PUFA on LCAT activity, reductions in CETP activity appear to relate to effects of alterations in the fatty acid moiety of cholesteryl esters and not to changes in CETP levels. Based on these data it would be predicted that intake of *n*-3 PUFA would have significant effects on reverse cholesterol transport and the intravascular conversions of plasma HDL subfractions.

5. LDL Receptor

Comparison of the effects of intake of commercial diets with 8% (by weight) fish oil versus safflower oil on binding of LDL to hepatic membranes suggested that the *n*-3 PUFA decreased expression of LDL receptors (Roach *et al.*, 1987). In a similar manner, studies of LDL binding to HepG2 cells preincubated with EPA indicated a significant inhibition of saturable LDL binding (Wong and Nestel, 1987); however, the same study reported that preincubation with linoleic acid also inhibited LDL binding compared to preincubation with oleic acid, a finding in contrast to other reports in the literature (Kuo *et al.*, 1990a).

In contrast to these findings, Tripodi *et al.* (1991) reported that the kinetic

parameters of LDL binding to rat hepatic membranes were altered by intake of 30% ene fish oil compared to 30% ene coconut oil, with the affinity constant K_d decreased (i.e., higher affinity) during intake of the *n*-3 fatty acids and the receptor number B_{max} unchanged, a finding independent of the source of tracer LDL. The apparent discrepancies between these reports no doubt relate to differences in the baseline comparison diet, either PUFA or SFA. Intake of PUFA has been shown to increase receptor number with no change in affinity (Fernandez and McNamara, 1988), whereas the *n*-3 PUFA effect apparently does not increase receptor number but increases the affinity. Under this situation, comparison to *n*-6 PUFA would result in a decrease in receptor binding of LDL while comparison to SFA would result in no difference. When the results of the study by Tripodi *et al.* (1991) are considered for *n*-3 PUFA-mediated changes in both LDL interaction with the receptor and changes in membrane receptor number, the data suggest that intake of fish oils actually reduces receptor number. The effects of *n*-6 and *n*-3 PUFA intake on LDL receptor expression are apparently different and more data are needed to determine how changes in membrane fluidity and in receptor expression combine to modify LDL receptor activity. This becomes apparent when considering the findings of Ventura *et al.* (1989) and Spady and Woollett (1990) of increased receptor-mediated clearance of plasma LDL in rats fed fish oil-containing diets as compared to safflower oil. A consensus on the effect of *n*-3 PUFA on LDL receptor number and expression cannot be reached at this time.

6. HDL Receptor Expression

There is one report of the effects of *n*-3 PUFA intake on hepatic HDL receptors indicating that, in contrast to the finding of reduced LDL receptor numbers, *n*-3 fatty acid intake increased rat hepatic HDL binding by 71% (Roach *et al.*, 1987). This observation supports data for increased hepatic HDL binding in guinea pigs fed 35% ene corn oil compared to lard (Fernandez and McNamara, 1991b), and suggests that increased membrane fluidity increases hepatic HDL binding. It would appear that with regard to the HDL receptor, *n*-3 PUFA act like *n*-6 PUFA, whereas for the LDL receptor, the effects could well be in opposite directions.

F. *n*-3 POLYUNSATURATED FATTY ACIDS AND ATHEROGENESIS

The question of whether *n*-3 PUFA are antiatherogenic has received considerable attention over the past years without any clear conclusions being drawn. Animal model studies have resulted in mixed results, with studies indicating reductions in indices of atherogenesis with fish oil intake in swine (Weiner *et al.*, 1986), rhesus monkeys (Davis *et al.*, 1987), Watanabe heritable

hyperlipidemic (WHHL) rabbits (Lichtenstein and Chobanian, 1990), and African green monkeys (Parks *et al.*, 1990b). In contrast, other studies in swine (Foxall and Shwaert, 1990), rabbits (Thiery and Seidel, 1987; Campos *et al.*, 1989), WHHL rabbits (Clubb *et al.*, 1989), and African green monkeys (Fincham *et al.*, 1991) have failed to find any evidence for an antiatherogenic effect of *n*-3 PUFA intake. Studies in rats (Rogers and Karnovsky, 1988) and swine (Foxall and Shwaert, 1990) have found increased monocyte adherence to endothelial cells with addition of *n*-3 PUFA to diets. Much of this confusion arises from the use of such extremely atherogenic diets in some models that it may be impossible to prevent atherosclerosis with dietary supplementation of *n*-3 PUFA, whereas substitution of dietary fat with fish oil effectively negates the atherogenic profile.

While the epidemiological data suggest that fish intake reduces CHD incidence, a study in coronary artery bypass patients failed to detect a protective effect of dietary supplementation with 3 g/day of *n*-3 PUFA on progression of atherosclerosis or rate of bypass occlusions (Masson *et al.*, 1990). It is unclear what role *n*-3 PUFA could play in determining atherogenesis or what dosages may be needed to obtain significant beneficial effects. The question of whether *n*-3 PUFA are protective against the development of CHD remains a topic of importance, debate, and uncertainty.

IX. DIETARY FATTY ACIDS, PLASMA LIPOPROTEIN LEVELS, AND GENETICS

Genetics plays a major role in determining plasma lipoprotein levels under any condition (Fisher *et al.*, 1989; Hopkins and Williams, 1989; Lusis, 1988; Sing and Moll, 1990) and contributes to the individual heterogeneity of responses to changes in the amount and type of dietary fat (Wolf and Grundy, 1983; McNamara *et al.*, 1987; Grundy and Vega, 1988). It has been estimated that as much as 50–60% of the interindividual variability of total, LDL, and HDL cholesterol is attributable to polygenic differences (Lusis, 1988). Genetic polymorphisms have been found for many of the enzymes and receptors involved in the processing of lipoproteins (reviewed in Lusis, 1988), and many of these polymorphisms contribute to increased plasma lipid levels. Genetic factors can alter the normal pattern of plasma lipoprotein metabolism, resulting in various forms of hyperlipoproteinemia, and have significant effects on CVD incidence in normolipidemic and hyperlipidemic individuals (Lusis, 1988). Clinical studies have demonstrated that many CHD patients have small LDL particles that are cholesteryl ester poor (Sniderman *et al.*, 1980; Teng *et al.*, 1983; Crouse *et al.*, 1985), resulting in an overproduction and increased catabolism of LDL (Kesaniemi and Grundy, 1983; Shi *et al.*, 1990), and this is an inherited pattern

associated with increased CHD risk (Austin *et al.*, 1988a,b). Other studies have shown relationships of CHD with increased postprandial lipoprotein levels and decreased postheparin LPL and HTGL activity (Groot *et al.*, 1991; Johansson *et al.*, 1991). As more studies investigate the role of alterations in lipoprotein structure and metabolism in control subjects and CHD patients, it becomes apparent that CHD risk arises from abnormalities not always readily apparent from analysis of fasting levels of plasma lipoproteins.

While the equations developed by Keys *et al.* (1965) and Hegsted *et al.* (1965) predict the average plasma cholesterol response to changes in dietary fatty acids, individual responses are highly variable (Wolf and Grundy, 1983; McNamara *et al.*, 1987; Grundy and Vega, 1988) and cannot be predicted (McNamara, 1987). Much of the reported variability in response to changes in either the amount or type of dietary fat results from significant heterogeneity of physiological regulatory responses in individuals in the population (McNamara *et al.*, 1987). Studies of plasma lipid levels and responses to high-fat, high-cholesterol diets in various mouse strains demonstrate that baseline plasma cholesterol levels and the change in levels resulting from the hypercholesterolemic diet are unique for each strain and that the response to diet cannot be predicted from baseline plasma cholesterol levels (Lusis *et al.*, 1987). The accumulated evidence from decades of clinical diet trials would suggest that a similar phenomenon occurs in humans. Two important considerations are that gene–environment interactions dictate not only the baseline plasma cholesterol level but also the precision of the homeostatic mechanisms maintaining that level (Hopkins and Williams, 1989).

Clinical studies indicate that variations in apolipoprotein structure affect not only plasma cholesterol levels and CVD risk but also the response to changes in dietary fatty acids. Isoforms of apoE; a structural protein of chylomicrons, VLDL, and HDL; and a ligand for hepatic lipoprotein receptors affect plasma total and LDL cholesterol levels (Davignon *et al.*, 1988), cholesterol absorption (Miettinen *et al.*, 1989), exogenous fat clearance (Weintraub *et al.*, 1987a,b), and the magnitude of the response to changes in dietary fat quality (Savolainen *et al.*, 1991; Tikkanen *et al.*, 1990a; Manttari *et al.*, 1991). The data suggest that effects of apoE phenotype may be influenced by dietary factors, including dietary fat quality and quantity and dietary cholesterol.

Analyses of genetic polymorphisms of plasma apolipoprotein genes have also been related to risk of CVD and to the extent of plasma lipoprotein changes with dietary fat modifications. The presence of certain types of restriction fragment-length polymorphisms (RFLPs) for the genes coding apoB, apoA-I, apoA-II, and apoE is involved in determining plasma lipoprotein levels and CVD risk (Berg, 1986; Demant *et al.*, 1988; Kessling *et al.*, 1988; Scott *et al.*, 1985; Wile *et al.*, 1989) and contributes to the response of plasma lipoproteins to changes in dietary fat quantity and quality (Xu *et al.*, 1990; Tikkanen *et al.*, 1990b).

Significant genetic influences on the extent of the plasma lipoprotein responses to changes in fat saturation and calories in humans complicate the issue of dietary recommendations and could in part explain some of the discrepant results in the literature when individual responses are considered. For example, in studies investigating the effects of changing from a typical American diet to a Step I diet, the data for individual patients demonstrate that some patients lower plasma LDL levels, some lower LDL and HDL levels, and some lower only HDL levels

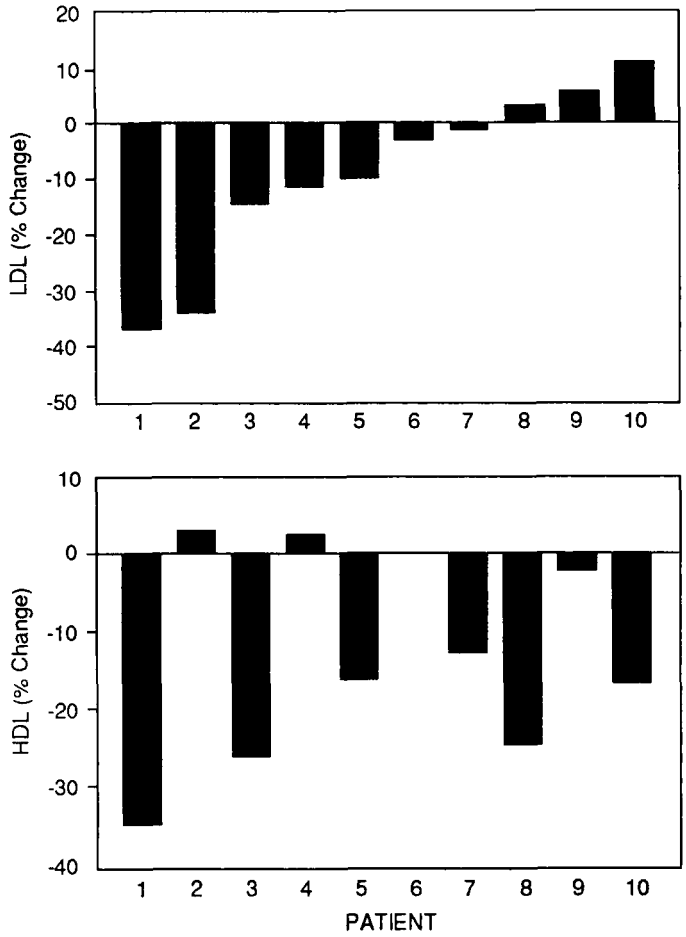

FIG. 8. Changes in plasma LDL and HDL cholesterol levels associated with a shift from an average American diet to a Step I diet. Patients (n = 10) were fed a 40% ene fat diet (P:M:S = 0.5:0.5:1.0) versus a 30% ene fat diet (P:M:S = 1:1:1) for x weeks, after which plasma LDL and HDL cholesterol levels were determined. Data presented as percent change in individual patients, illustrating the heterogeneity of responses to dietary fat interventions. (After Wolf and Grundy, 1983.)

(Fig. 8) (Wolf and Grundy, 1983). Such a range of responses to the same intervention no doubt has a strong genetic basis and raises the question of who will and who will not benefit from dietary interventions when they are implemented on a population basis. A better understanding of the distribution of genetic patterns in the population, their impact on CVD risk, and their contribution to the efficacy of dietary interventions is a major area of investigation and will significantly expand our understanding of the diet–heart disease relationships.

X. DIETARY FATTY ACIDS AND CARDIOVASCULAR DISEASE

From the studies presented in this review it is clear that dietary SFA have a significant hypercholesterolemic effect in most individuals and that reductions in calories derived from SFA lower plasma total and LDL cholesterol levels in humans. It is also clear that modest reductions in LDL cholesterol can be achieved by dietary fat changes without necessarily lowering HDL cholesterol levels. The ultimate question, however, is whether such changes will result in a reduced incidence of CHD in the population and whether they will do so without side effects. The evidence from dietary and drug trials testing the effect of lowering plasma cholesterol on CHD incidence and mortality indicates that for every 1% reduction in plasma cholesterol there is a 2% reduction in CHD over 6 years (Holme, 1990). Epidemiological studies also indicate that a 1% lower HDL level is associated with a 3–4% increase in CHD risk, even when total cholesterol levels are less than 200 mg/dl (Wilson, 1990). Based on these considerations, modest changes in dietary SFA intake would be expected to lower CVD risk by lowering plasma LDL cholesterol levels in most individuals; the question is, how much lowering and what reduction in CHD risk. It can be estimated from the equations of Keys and Hegsted (Table IV) that changing from the current American diet to one of 30% ene fat, with equal proportions of SFA, MUFA, and PUFA, that plasma total cholesterol levels would on average decrease by 14 mg/dl. For someone with an average cholesterol of 210 mg/dl, this equates to a 6.7% decrease and would in theory lower CHD risk by 13%.

At this point it is well to ask what a 13% reduction in CHD relative risk represents, since it means different things to different people. From a public health perspective, a 13% reduction is highly desirable and would have a significant impact on CHD morbidity and mortality rates in the population. For the individual this is less of a concern relative to knowing their own individual risk reduction. On an individual basis, the first consideration deals with an assessment of not only the individual's plasma total and lipoprotein cholesterol levels, but also other risk factors, such as elevated blood pressure, cigarette smoking, and obesity, since effective risk reduction requires that all CHD risk factors be treated. Assuming other life-style risk factors are minimal, one can estimate the benefits of lifelong adherence to a plasma cholesterol-lowering diet based on models developed from epidemiological and clinical studies.

Using risk models, studies have attempted to estimate the benefits of dietary interventions on life expectancy (Taylor *et al.*, 1987; Malenka and Baron, 1989; Browner *et al.*, 1991). Taylor *et al.* (1987) estimated that for persons between the ages of 20 and 60 years, lifelong adherence to a plasma cholesterol-lowering, fat-modified diet would result in a gain in life expectancy of 3 days to 3 months for low-risk individuals and 18 days to 12 months for those at high risk. Browner *et al.* (1991) estimated that a population-wide reduction in fat calories from 37 to 30% would result in 42,000 of the 2.3 million deaths per year being deferred and that the average gain in life expectancy would be 3 to 4 months, chiefly in those over 65 years of age. It is obvious that on a population basis, intervention has a significant impact (a 3-month increase of life expectancy times 240 million results in 60 million years of additional life); however, on an individual basis the gain of an additional 3 months might be considered insignificant. These estimates do not, however, address effects of such dietary changes on the quality of life and overall well being; no data are available to estimate these potential benefits. In all of these estimates of potential gains accrued by dietary interventions, the life expectancy gains are primarily for those with plasma cholesterol levels in the borderline high and high-risk categories, with little if any benefit for those individuals having average CHD risk based on plasma cholesterol concentrations of less than 200 mg/dl. If one adds the variables of the heterogeneity of responses to dietary interventions and genetic factors, which increase CHD risk and are unaffected by dietary intervention, it becomes virtually impossible to predict who will and who will not benefit from a reduction in SFA calories.

Analyses of the effects of dietary intervention studies on CHD incidence and total mortality suggest that interventions can lower CHD incidence; however, total mortality rates are usually unaffected (McCormick and Skrabanek, 1988; Holme, 1990; Roussouw and Rifkind, 1990). The same pattern holds true for most of the hypolipidemic drug trials, showing decreased CHD incidence with no change in total mortality (Holme, 1990; Roussouw and Rifkind, 1990). There is great debate regarding these observations and what the cause and effect relationships may be (Oliver, 1991); what is obvious from a number of studies is that the relationship between plasma cholesterol levels and total mortality is a J-shaped curve, with increased mortality at both the lower and upper ends of the distribution (Martin *et al.*, 1986). At very low levels of plasma cholesterol (<160 mg/dl) there are indications of an association with increased deaths due to cancer, cerebral hemorrhage, and other causes (Roussouw and Rifkind, 1990). Caution needs to be exercised in interpretation of these data on either side of the question, and only by detailed follow-up of patients in clinical trials will a better understanding of these relationships be gained.

What effect then will reducing total fat and SFA calories have on plasma cholesterol levels and CVD incidence in the population? It is reasonable to assume

that some degree of risk reduction will be achieved by modifying the amount and type of fat in the diet and that the proposed dietary modifications will have little if any negative effects on nutritional balance. In large part this is true because the recommended dietary changes are not extremes and represent the balance and moderation essential for overall nutritional well-being. Some of the more radical dietary changes proposed by extremists do have the risk of nutritional imbalance and can often negate a serious attempt at CVD risk modification due to problems of compliance.

One benefit often gained from reducing total fat in the diet is a decrease in caloric intake, with the eventual outcome a reduction in the extent of obesity in the population. Reducing abnormalities of cholesterol and lipoprotein metabolism associated with obesity (McNamara, 1987) would be a very effective CVD risk reduction modality associated with reductions in a number of risk factors: hypertension, low HDL levels, glucose intolerance, and obesity itself. While changes in dietary fat intake may have a relatively modest effect on plasma cholesterol levels per se, the modifications of CVD risk factors by decreasing excess body weight could be substantial. An additional benefit would also be gained from increased fiber intake associated with a low-fat diet.

XI. SUMMARY

Dietary fat quality and quantity significantly affect the metabolism of all the plasma lipoproteins and probably constitute the most significant dietary determinants of plasma lipoprotein levels. Since the major role of the plasma lipoproteins is the transport of exogenous and endogenous fat, this would be expected of a highly regulated, metabolically homeostatic system. The data clearly show that dietary fat saturation affects all aspects of lipoprotein metabolism, from synthesis to intravascular remodeling and exchanges to receptor-mediated and nonspecific catabolism. The experimental data regarding dietary fatty acid effects on lipoprotein metabolism are complicated and at times contradictory due to the large degree of metabolic heterogeneity in the population, which, when coupled with the known abnormalities of lipoprotein metabolism associated with certain types of hyperlipoproteinemia, can present responses from A to Z. It is clear that the same dietary pattern has different effects in different individuals and that complicating factors of individuality raise some concerns regarding generalized dietary recommendations. As new knowledge of the role of dietary factors and CVD risk develops, and our abilities to characterize the individual patient's response to dietary interventions become more refined, it may be possible to specify dietary fat intervention from a patient-oriented concept rather than a single all-purpose diet approach. Thus it would be possible to design dietary interventions to match patient needs and gain both efficacy and compliance. With

the spectrum of approaches possible—low fat, moderate fat with MUFA, *n*-3 PUFA, etc.—we should be able to approach dietary interventions to reduce CVD risk at both a population-based level and a patient-specific level.

There remains much to learn regarding the effects of dietary fatty acids on the synthesis, intravascular modifications, and eventual catabolism of the plasma lipoproteins. The area of lipoprotein metabolism in health and disease, of its modifications by diets and drugs, and of the contributions of genetic heterogeneity to these processes is one of notable advances over the past two decades and continues to be an area of intense investigation.

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