Flavor Protein Interactions. Binding of Carbonyls to Bovine Serum Albumin: Thermodynamic and Conformational Effects

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The binding of 2-heptanone, 2-nonanone, and nonanal to bovine serum albumin was studied. Thermodynamic analysis revealed that the affinity of ketones for bovine serum albumin increased with chain length. An increment in the chain length by one methylene group increased the binding affinity threefold, indicating that the binding is hydrophobic in nature. Temperature did not have any appreciable effect on binding, suggesting that the enthalpy of binding is negligible and the thermodynamic driving force is entropic in origin. Nonanal bound to bovine serum albumin more tightly than 2-nonanone. Binding of 2-nonanone induced conformational changes in bovine serum albumin as revealed from absorption and fluorescence studies. The possible implications of structural changes in food proteins is discussed.

Foods are complex multicomponent systems and the interactions of flavors with the different classes of compounds significantly affect their impact on flavor perception (Maier, 1970; Solms et al., 1973; Franzen and Kinsella, 1975). The perceived flavor of foods is significantly affected by the differences in rates and extents of flavor release when food is chewed. According to McNulty and Karel (1973), the concentration of the flavor in the aqueous saliva determines flavor perception. Because the perceived flavor is ultimately most important in determining food acceptibility, the phenomenon of flavor binding and release is extremely significant. Solms et al. (1973) reviewed the information on the interactions of flavors with food components.

The binding of flavors to food components, especially proteins, which may suppress their primary flavor impact, is a problem that was recognized as technologists tried to fabricate food analogues and new foods from novel proteins (Kinsella, 1978). Proteins have little flavor, but they influence perceived flavor because they may contain bound off-flavors, and they can modify flavors by selective binding of flavorants.

Information concerning the binding of flavors to proteins is very limited (Gremli, 1974; Franzen and Kinsella, 1974). In order to facilitate the successful flavoring of food analogues and develop practical methods for the removal of off-flavors, the mechanism(s) and thermodynamics of flavor binding need to be understood. Research in this area is limited by the methods currently available. However, using the rationale and information from studies of protein–ligand interactions we studied the interactions between ketones and bovine serum albumin. Bovine serum albumin (BSA) was selected because its molecular properties and binding characteristics are reasonably well described (Steinhardt and Reynolds, 1969).

EXPERIMENTAL SECTION

Materials and Methods. Crystallized and lyophilized bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO) (Lot No. 98C-8050). Fatty acid impurities were removed by charcoal treatment (Chen, 1967). BSA thus treated contained about 0.4 mol of free fatty acid per mole of BSA as determined by the method of Dole (1956). Spectral grade heptane was purchased from Fisher Scientific Co. (Fair Lawn, NJ); 2-nonanone (99+%), 2-heptanone (98%), and nonanal (98%) were obtained from Aldrich Chemicals (Milwaukee, WI). All other chemicals used in the study were of reagent grade. Distilled and deionized water was used in all the experiments.

BSA Solutions. An absorptivity of 6.67 at 279 nm for 1% solution was used for determining the BSA concentration (Leonard and Foster, 1961). BSA solutions at concentrations of 0.6% were made in 20 mM phosphate buffer, pH 7.0 containing 0.15 M NaCl. Sodium azide (0.02%) was used in all the experiments to prevent microbial growth.

Gas Chromatography. A Perkin-Elmer Model 900 gas chromatograph equipped with flame ionization detector was used. Stainless steel column ($^1/_8$ in. diameter and 10 ft length) packed with apiezon (10%) coated on chromosorb was used. The gas flow rates were as follows: hydrogen, 15 mL/min; oxygen, 300 mL/min; and nitrogen, 40 mL/min. For 2-nonanone and nonanal the column temperature was 120 °C and for 2-heptanone 70 °C was used.

Measurement of Binding. Liquid–liquid partition equilibrium method (Spector et al., 1969; Ali Mohammadzadeh-K et al., 1967) was employed to study the binding of ketones and aldehyde to BSA. In a typical experiment, 2 mL of BSA solution was taken in a flat-bottom glass vial. A sampling tube was placed inside the vial. The heptane phase (0.25 mL) containing a known amount of the flavor ligand was placed gently on the aqueous surface surrounding the sampling tube. The vials were capped tightly with Teflon-lined screw caps and shaken gently in an incubator (New Brunswick, NJ) at 25 °C for about 36 h to attain equilibrium. The time required for equilibration was determined for each study. After equilibration, 1 mL of the aqueous phase was drawn out through the sampling tube using a syringe and placed in a vial containing 1 mL of heptane. The sampling tube facilitated removal of the aqueous phase without touching the heptane phase. The vials were capped tightly with Teflon-lined screw caps and shaken horizontally for about 2 h. Duplicate controls, devoid of protein, were equilibrated and extracted under identical conditions. Since the partition coefficient of the ketones and the aldehydes studied were of the order of 10^{-3} to 10^{-4}, essentially all the ligand from the aqueous phase was extracted into the heptane phase. In fact, a second extraction of the aqueous phase did not yield any of the ligand. The concentration of the ligand in the heptane extract was determined by gas chromatography. The difference in the concentration of the ligand between the controls and samples containing BSA represented the amount of ligand bound to protein. Using a molecular weight of 67 000 for BSA, the number of moles of ligand bound per mole of protein (ν) was cal-

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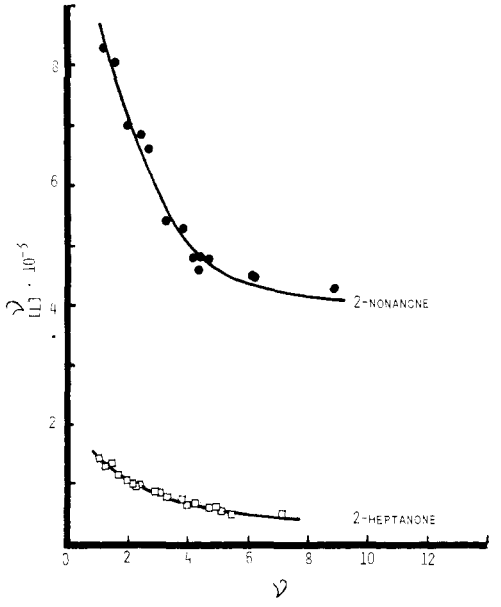

Figure 1. Scatchard plots for the binding of 2-nonanone and 2-heptanone to BSA at 25 °C. ν is the moles of ligand bound per mole of BSA and [L] is the unbound ligand concentration. BSA solution (0.6%) was made in 0.02 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl.

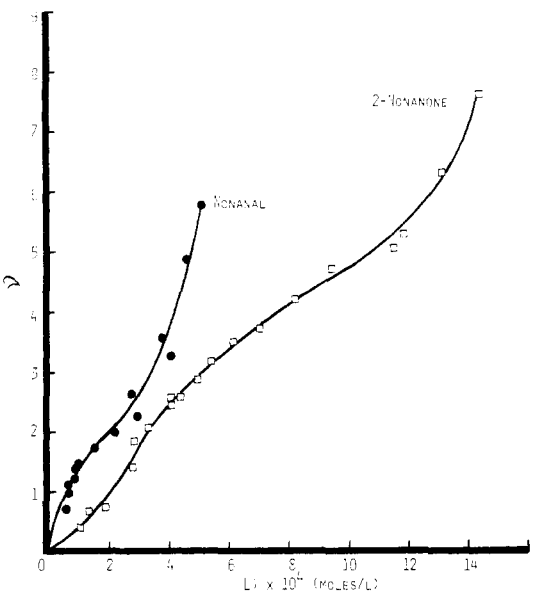

Figure 2. Binding isotherms for the binding of 2-nonanone and 2-nonanal to BSA at 25 °C. BSA (0.6%) was in 0.02 M sodium phosphate buffer, pH. 7.0, containing 0.15 M NaCl.

culated. Ligand concentration in the controls were taken as the free ligand concentration ([L]).

Treatment of the Data. The data were analyzed by the method of Scatchard (1949), i.e., by plotting ν, the number of moles of ligand bound per mole of protein, against $\nu/[L]$, where [L] is the free ligand concentration. According to Scatchard (1949)

$$\nu/[L] = nK - \nu K$$

where n and K are the total number of binding sites and the intrinsic binding constant, respectively. The value of n was calculated by extrapolating the initial slope to meet the abscissa.

Difference Spectra. Difference spectra of BSA were measured in a Perkin-Elmer Model 356 double-beam spectrophotometer. Quartz cells of 2-mm path length were used. The protein concentration was 0.6%.

Fluorescence Spectra. The fluorescence spectra of native BSA and ligand-treated BSA solutions were measured in a Perkin-Elmer Model 203 fluorescence spectrophotometer. The protein solutions were diluted with the corresponding controls to obtain a concentration of 0.1 OD at 279 nm. Dilution with the corresponding controls was necessary to maintain the same free ligand concentration after dilution, which otherwise would result in change in the ν values. The protein solutions were excited at 280 nm and the emission intensities were measured from 250 to 400 nm.

***N*-Bromosuccinimide (NBS) Titration with BSA.** BSA at concentration of 0.08% in 0.15 M NaCl, pH 3.0, was equilibrated (4 mL) with heptane phase (0.5 mL) containing a known amount of 2-nonanone for 36 h at 25 °C. After equilibration, the amount of ligand bound to BSA and the unbound ligand concentration were determined as before. The protein solution (2 mL) was placed in a cuvette, and microliter aliquots of 9 mM *N*-bromosuccinimide (in water) stock solution were added and the solution stirred with a small magnetic bar placed inside the cuvette. The absorbance of the protein solution at 279 nm was measured for each addition of *N*-bromosuccinimide. Titration of *N*-bromosuccinimide with control BSA solution (equilibrated with heptane containing no 2-nonanone) was done in the same way.

RESULTS AND DISCUSSION

Effect of Chain Length. The Scatchard plots for the binding of 2-heptanone and 2-nonanone to BSA were nonlinear (Figure 1), indicating cooperative binding behavior. Extrapolation of the initial slopes to intercept the abscissa gave a value of about 6, suggesting that BSA has about five–six initial binding sites for both 2-heptanone and 2-nonanone. This value is in agreement with the reported number of binding sites in BSA for some neutral ligands (Steinhardt and Reynolds, 1969) and free fatty acids (Spector, 1975). The binding constants obtained from the initial slopes were 2.7×10^2 and 1.8×10^3 L/mol, respectively. An increase in the chain length by two methylene groups increased the binding constant about sixfold. The corresponding standard free energy change of binding of 2-heptanone and 2-nonanone were −3.3 and −4.4 kcal/mol at 25 °C. This gave a value of about −0.55 kcal per methylene group. The effect of chain length on the free energy of interaction suggests that the binding is hydrophobic in nature. The magnitude of this effect is such that the protein–ligand interaction is favored by about −550 cal for each increment of one methylene group in the homologous series of methyl ketones. Assuming that the contribution to the hydrophobic free energy by keto group, which is relatively polar, is negligible, the free energy of interaction between 2-heptanone and BSA can be considered to be only due to six methylene groups. On the basis of this, the theoretical hydrophobic free energy of interaction of 2-heptanone to BSA would be 3.3 kcal (6 × 550 cal). Similarly for 2-nonanone it would be 4.4 kcal (8 × 550 cal). In fact, experimentally observed hydrophobic free energy of interaction of 2-heptanone and 2-nonanone to BSA were 3.3 and 4.4 kcal/mol, suggesting that the keto group does not contribute to the hydrophobic free energy.

Effect of Functional Group. The binding isotherms for 2-nonanone and nonanal to BSA are shown in Figure 2. Nonanal exhibited a higher binding affinity to BSA than 2-nonanone. The difference in the binding affinities may be due to the position of the keto group in the chain. In the case of 2-nonanone, the presence of keto group at the 2-position may introduce a steric hindrance for the

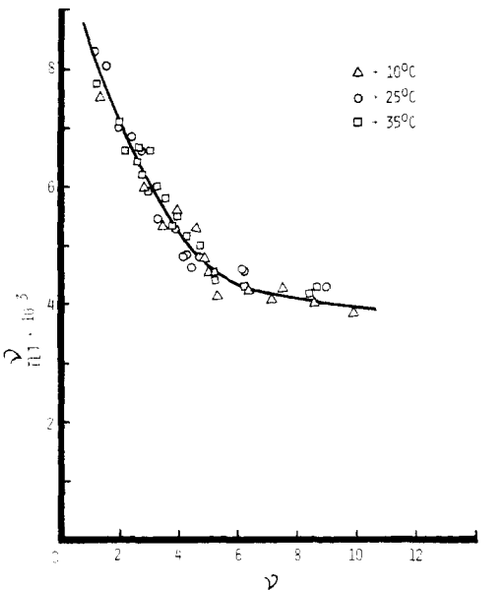

Figure 3. Effect of temperature on the Scatchard plot for the binding of 2-nonanone to BSA. BSA solutions (0.6%) were made in 0.02 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl.

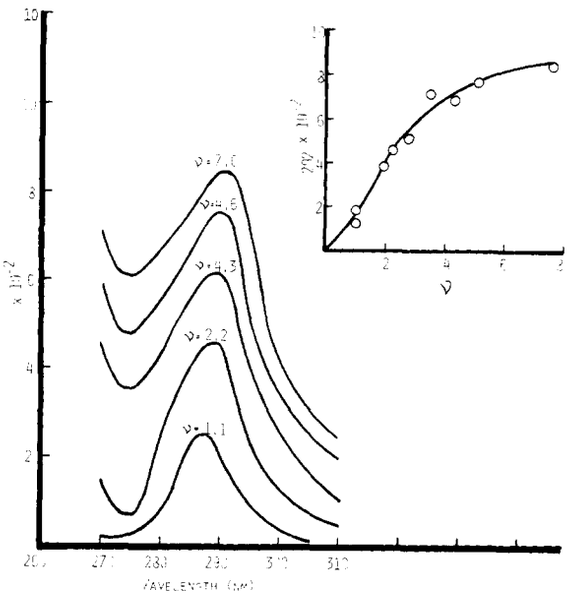

Figure 4. Difference spectra of BSA at various molal ratios of binding of 2-nonanone. The inset shows the difference in absorbance at 292 nm as a function of molal ratio of binding ν.

hydrophobic affinity for the chain as a whole. Whereas in the case of nonanal in which the aldehyde group is in the terminal 1-position, the steric hindrance to hydrophobic interaction would be less compared to ketones. We suggest that such steric hindrance would result in decrease in the free energy of interaction contributed by methylene groups in ketones compared to aldehydes.

The observed difference in the binding affinities of 2-nonanone and nonanal to BSA may also be due to their chemical nature per se. Since aldehydes are more reactive than the ketones, they may possibly react with the ϵ-amino groups which would be reflected in an apparent higher hydrophobic affinity. However, upon analysis of the nonanal–protein complex, there was no reduction in the available ϵ-amino groups of BSA as determined by the (2,4,6-trinitrobenzene)sulfonic acid (TNBS) method (Hall et al., 1973). The possibility of nonanal forming hydrogen bonding with the functional groups in BSA, however, is conceivable.

Effect of Temperature. Temperature had little effect on the binding of 2-nonanone to BSA (Figure 3). In other words, the change in enthalpy, ΔH, for the binding process is negligible. According to classical thermodynamics, ΔG, $= \Delta H - T\Delta S$. Since ΔH is very small, the favorable free energy change must be due to increase in the entropy of the system. In fact, it has been established that the interactions which are hydrophobically motivated have practically zero enthalpy change and the driving force is predominantly entropic in origin (Lewin, 1974; Klotz and Franzen, 1962). Hence $-RT \ln K = -T\Delta S°$ or $\Delta S° = R \ln K$. Assuming that the enthalpy change is zero, the entropy change ΔS for the 2-nonanone to BSA interaction was 14.7 cal mol^{-1} K^{-1}. The positive entropy change associated with the binding may be due to the structural changes in the solvent (water) when solute–solvent interactions are replaced by solute–solute interactions and also be due to the disorientation and unfolding of the protein molecule (Steinhardt and Reynolds, 1969). To determine if binding causes structural changes, we monitored conformational changes in the BSA as binding occurred.

Structural Changes in BSA. The nonlinearity of Scatchard plot (Figure 1) for the binding of 2-heptanone and 2-nonanone which is reflected in the sigmoidal behavior of the binding isotherms (Figure 2) suggests that binding is cooperative. In the case of 2-nonanone, the Scatchard plot exhibited straight line up to about 4 molal ratios of binding, and above this the slope changed drastically. This suggested that there are two classes of binding sites in BSA for 2-nonanone. The first six sites (obtained by extrapolating the initial slope) have higher affinities compared to the second class of binding sites. It is difficult to estimate the number of weaker binding sites because of the uncertainty involved in extrapolation. It is possible that binding of the ligand to the initial sites might be responsible for the creation of additional weaker binding sites due to conformational changes in the protein (Karush, 1950, 1954). The structural changes in BSA, which are responsible for the cooperative behavior, upon ligand binding can be detected from the changes in UV absorption and fluorescence emission behavior of BSA.

The difference spectra of BSA at various molal ratios of binding of 2-nonanone are shown in Figure 4. The peak observed at 287 nm at low molal ratios of binding of 2-nonanone shifted gradually to 292 nm at higher molal ratios of binding. This suggested that at low molal ratios of binding (ν = 1–2), the increase in absorbance was primarily attributable to the ionization of tyrosine residues (Donovan, 1969). At higher molal ratios of binding, the 287-nm peak might have been masked by the exposure of previously buried tryptophan residues which have characteristic peak at 292 nm (Donavan, 1969; Polet and Steinhardt, 1968). This is further evidenced by the fluorescence emission behavior of BSA upon binding of 2-nonanone (Figure 5). The inset in Figure 5 shows the percent fluorescence emission intensity at 340 nm as a function of number of moles of 2-nonanone bound per mole of BSA. The quenching of fluorescence upon binding of 2-nonanone is due to exposure of tryptophan residues to the surrounding polar solvent as a result of conformational changes in the protein.

Conformational changes in BSA can also be monitored by *N*-bromosuccinimide titration method (Spande and Witkop, 1967). *N*-Bromosuccinimide (NBS) reacts with tryptophan residues in proteins and this reaction is followed conveniently by measuring the drop in optical

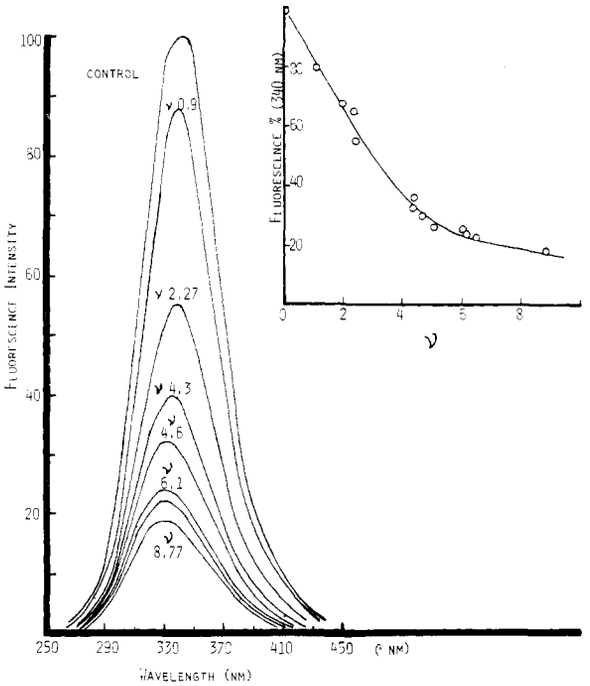

Figure 5. Fluorescence spectra of BSA at various molal ratios of binding of 2-nonanone: BSA concentration was about 0.1 OD at 279 nm. Buffer: 0.02 M phosphate buffer, pH 7.0, containing 0.15 M NaCl. The inset shows the percent fluorescence intensity relative at 340 nm as a function of molal ratio of binding of 2-nonanone. The excitation wavelength was 280 nm.

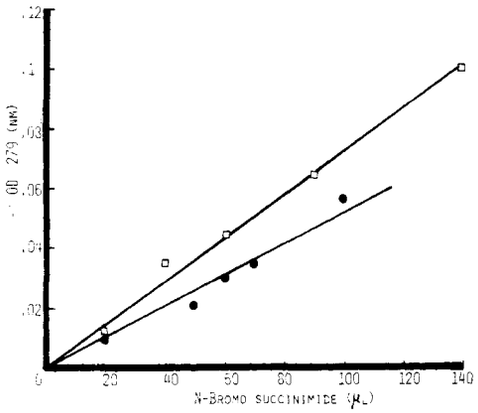

Figure 6. Titration of *N*-bromosuccinimide with BSA: protein concentration was 0.54 OD at 279 nm. *N*-Bromosuccinimide was added in microliter aliquots from a stock solution of 9 mM. BSA solution was made in 0.15 M NaCl, pH 3.0. (□–□) Native BSA; (●–●) BSA treated with 2-nonanone (ν = 5).

density of the protein solution at 280 nm. The titration of NBS with native and 2-nonanone treated BSA is shown in Figure 6. The decrease in the optical density with NBS concentration was higher in the case of 2-nonanone-treated BSA (ν = 5.0) than in the case of native BSA. It has been reported that some proteins react very slowly with NBS compared to others (Ramachandran, 1960) and this behavior has been attributed to the buried tryptophan residues in the hydrophobic regions of some proteins which are not readily accessible to NBS (Witkop, 1961). The observed difference in the reactivity of tryptophan residues with NBS in the case of native and 2-nonanone-treated BSA may be due to the unfolding of the hydrophobic sites upon 2-nonanone binding which exposes the previously buried tryptophan residues which are then readily available for NBS. This is in agreement with the fluorescence data in which the quenching of fluorescence is due to the

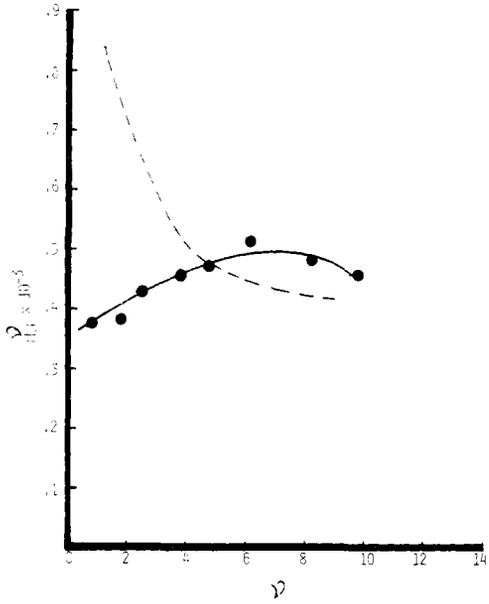

Figure 7. Effect of dithiothreitol on binding of 2-nonanone to BSA: BSA (0.6%) was in 0.02 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl. (●–●) Scatchard plot in the presence of 1 mM dithiothreitol; (– –) binding in the absence of dithiothreitol.

exposure of tryptophan residues to the solvent environment.

Effect of Thiol Reducing Agents on Binding. BSA contains 17 disulfide bridges (Schachman, 1963). The disulfide bridges are partly responsible for the native structure of BSA. If the initial number of strong binding sites in BSA is due to its unique structural properties, then reduction of disulfides with thiol reagents would alter the conformation of these sites and this should be reflected in the binding behavior of 2-nonanone. The Scatchard plot for the binding of 2-nonanone to BSA in the presence of 1 mM dithiothreitol is shown in Figure 7. There is drastic change in binding behavior which suggests that the reduction of disulfide bridges and subsequent structural modification of the BSA changed the affinity of the binding sites for 2-nonanone.

The above findings suggest that the binding affinity of carbonyls to BSA is fairly strong and is influenced by the chain length, the functional groups and the structural state of the protein itself. By modifying the structure of the protein, the binding affinity of carbonyls to proteins can be manipulated. The results obtained for BSA, which is a single polypeptide, may not be the same for oligomeric proteins like soy proteins. For example, the effect of temperature on the dissociation/association of subunits of oligomeric proteins would also result in the temperature dependency of binding of carbonyls. Solms et al. (1973) reported that the binding isotherms for butanal and butanone to soy proteins were dependent on the protein concentration. The binding affinities were much greater at low protein concentrations. This might be due to protein–protein interaction at higher concentrations which would result in the apparent decrease in the protein–ligand interaction. The structural changes in BSA upon binding of low amounts of carbonyls suggests that such changes could also be induced in soy proteins by hexanol and hexanal binding (Arai et al., 1970) which, in addition to the flavor acceptability, would affect the functional properties of these proteins (Kinsella, 1979). These observations are very relevant to soy proteins where carbonyls generated by lipoxygenase upon initial breakage of the seed

may bind to the proteins and cause conformational changes which affect the physical properties of the isolated proteins.

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Detoxification of Jojoba Meal

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Jojoba meal, after removal of the oil from the seeds of *Simmondsia chinensis*, contains up to 30% crude protein, making it a potential animal feed ingredient. This meal is toxic to rodents and chickens and less toxic to sheep. Toxicity is due to the presence of simmondsin and as many as three other structurally related cyanomethylenecyclohexyl glycosides in the meal. Solvent extraction and heat and chemical methods were investigated for detoxifying the meal. A new method was devised to hydrate the cyano compounds in the meal rapidly to amides using ammoniacal hydrogen peroxide. This detoxified jojoba meal was well accepted by mice, chickens, and sheep at 10% additive levels in rations. Toxicity of simmondsin is apparently related to the cyano aglycon which can be split from the glucose conjugate in the gut or stomach of monogastric animals. Microorganisms in the rumen of sheep serve to detoxify simmondsin in jojoba meal.

Jojoba is an oilseed shrub that grows naturally on arid lands in the southwest United States and in Mexico. Several thousand acres have been planted in California and Arizona as the basis for a new economic crop. The principal product of this new industry is an unusual oil comprised of esters of long-chain monounsaturated acids and alcohols with structures similar to sperm oil, a commodity no longer available in the United States. After removing the jojoba oil from jojoba seeds, the remaining meal is high in protein and is a potential livestock feed ingredient (Verbiscar and Banigan, 1978a,b).

The term jojoba meal is used here to mean the plant material remaining after the seeds containing some hulls have been processed to remove jojoba oil. Jojoba seed meal is the residual material left after deoiling completely dehulled seeds. The jojoba meal that we have used throughout this study contains hull material. The presence of hulls in the seeds facilitates expression of jojoba oil but

Table I. Composition of Jojoba Seed Meal, Jojoba Meal, and Hulls[a]

component	seed meal no. 377,[b] %	meal no. 377,[c] %	meal no. 278,[d] %	seed hulls, %
crude protein, N × 6.25	29.1	24.1	20.1	7.0
moisture	8.9	5.8	5.7	10.7
crude oil (ether extract)	3.0	1.6	0.9	0.7
crude fiber	8.1	11.0	14.3	15.6
ash	3.1	4.9	3.6	4.4
total sugars	8.8		6.4	3.3
simmondsin (I)	5.2	4.2	3.6	0.2
simmondsin 2′-ferulate (II)	1.5	0.5	1.3	0

[a] Products courtesy of San Carlos Apache Jojoba Development Project, San Carlos, AZ. [b] Hexane extracted hulled seed meal. [c] Methylene chloride extracted meal from seeds with ~8.5% hulls. [d] Hexane extracted meal from seeds with ~17% hulls.

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