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Binding of volatile flavor compounds to purified soy proteins in an aqueous model system

by

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INTRODUCTION

There are a number of problems that prevent soybeans from reaching their potential as a protein source for humans. Maga (1973) has pointed out four of these problems: 1. many soy products are foreign to domestic food consumption patterns; 2. the "health food image" is deleterious to widespread use; 3. legal implications restrict usage and; 4. the characteristic soy flavor limits incorporation. The off-flavor problem often limits acceptance of soy products and restricts the use of isolated soy proteins and soy protein concentrates (Kinsella and Damodaran, 1980). The compounds responsible for the off-flavor belong to a number of chemical classes including ketones, aldehydes, alcohols, furans, phenolics and amines. The most commonly encountered flavor is beany-grassy and the contributing compounds include n-hexanol, hexanal, 3-cis-hexenal, n-pentyl furan and ethyl vinyl ketone (Rackis et al., 1979). The flavor compounds are produced by the action of lipoxygenase and once bound to the protein are difficult to remove. Interactions can take place between the flavor compound and protein that lead to a change in the character of the flavor. Addition of spices may cause dramatic changes in the flavor profile and lead to formation of new off-flavors (Schutte and van den Ouweland, 1979).

Commercial methods of dealing with the off-flavors are not satisfactory. At present, removal of the flavor compounds which are associated with the soy protein cannot be done without seriously

imparing functional properties of the proteins. Functional properties, such as solubility, viscosity, water binding, emulsification, and foaming are necessary for the incorporation of soy protein into food systems (Kinsella, 1979). Knowledge of the forces involved in the binding may make it possible to remove the flavor compounds more easily. At present, no thorough study has been undertaken using highly purified protein in a model system devoid of reducing agents and antimicrobial compounds.

This study was initiated to investigate the nature of the binding between soy proteins and the off-flavor compounds.

LITERATURE REVIEW

Soybean Proteins

Soybeans contain a number of different proteins that include trypsin inhibitors, enzymes including lipoxygenase, and storage proteins (Nielsen, 1985a). Older literature described the storage proteins by their Svedberg sedementation coefficients. The two main storage proteins are glycinin (11S) and B-conglycinin (7S). Both of these proteins are oligomers and have no physiological function other than as stores of amino acids for use during seed germination.

Glycinin has been reported to consist of two major classes of subunits, acidic and basic, which exist as acidic-basic pairs joined by disulfide bonds (Nielsen, 1985a,b). At least 6 acidic and 4 basic subunits have been reported (Brooks and Morr, 1985). There is clear heterogeneity in glycinin composition from different cultivars (Kitamura et al., 1980), and gelling characteristics of glycinin from different cultivars have been linked to differences in subunit composition (Nakamura et al., 1984). The acidic subunits have molecular weights ranging from 10 to 45 KDaltons. Basic subunits range from 19 to 23 KDaltons. The acidic (A) and basic (B) subunits are not randomly paired, rather they are synthesized from a single mRNA and subsequently cleaved (Nielsen, 1985a,b). Most of the pairs are covalently linked via disulfide bonds. The currently accepted pairings are described by Brooks and Morr (1985). Subunit A4 is not covalently linked but is associated with A5. The native structure

seems to be a dodecamer, made up of six A-B pairs (Badley et al., 1975). At pH 7.6, when ionic strength is decreased from 0.5 to 0.1, the 11S fraction forms association polymers (dodecamers) but below ionic strength 0.1, the dodecamer may dissociate into half-11S hexamers (Koshiyama, 1972; Utsumi et al., 1987).

B-Conglycinin consists of at least three different subunits: a, a', and b; gamma and delta subunits have also been reported (Thanh et al., 1975, Thanh and Shibasaki, 1976b, 1977, 1978; Yamauchi et al., 1981). The subunits have been reported to be randomly associated, with all possible combinations existing. The native structure seems to be a hexamer (Koshiyama and Fukushima, 1976; Iibuchi and Imahori, 1978a,b). The hexamer structure may dissociate into two trimers at high ionic strength (>0.5) or acid (<pH 4.8) conditions (Koshiyama, 1968; Thanh and Shibasaki, 1979).

A number of different methods have been described for the purification of glycinin and B-conglycinin (Badley et al., 1975; Catsimpoolas and Meyer, 1968; Hill and Breidenbach, 1974; Kitamura et al., 1974; Koshiyama and Fukushima, 1976; Lambert et al., 1987; Rao and Rao, 1977; Thanh et al., 1975; Thanh and Shibasaki, 1976a). The most common method of preparing crude proteins was reported by Thanh and Shibasaki (1976a) and is based on differences in isoelectric points of the two proteins in dilute Tris buffer. The method of Kitamura et al. (1974) which uses gel filtration and affinity chromatography is commonly applied to the crude fractions, obtained by using Thanh and Shibasaki's method, to produce highly pure proteins.

A recent publication indicated that a combination of the solubility method of Thanh and Shibasaki (1976a) and ammonium sulfate fractionation produced proteins which were highly pure by immunological analysis (Iwabuchi and Yamauchi, 1987). The crude protein fractions produced from the method of Thanh and Shibasaki were reported to be 79 and 52% pure for glycinin and B-conglycinin respectively as determined by an immunological method (Iwabuchi and Yamauchi, 1987), but the purities described by Thanh and Shibasaki are much greater.

The glycinin content of 10 varieties of soybeans has been reported by Hughes and Murphy (1983) to range from 31.4-38.3 % of the total protein. The technique used was sodium dodecyl sulfate polyacrylamide gel electrophoresis with cutting out of the glycinin bands and quantitation by using Coomassie blue dye. Murphy and Resurreccion (1984) used an immunological method to examine the B-conglycinin and glycinin contents of 12 soybean varieties. The B-conglycinin and glycinin contents ranged from 15.2 - 20.9 and 38.2 - 57.4 % of the protein, respectively. The difference between the Hughes and Murphy (1983) and the Murphy and Resurreccion (1984) data was presumably due to the greater specificity of the latter study. Iwabuchi and Yamauchi (1987) found that the one sample of soybeans that they examined had 32 % glycinin by using a radial immunodiffusion technique. The freeze-drying of soy protein, used by Iwabuchi and Yamauchi (1987), has been reported to decrease the sulfhydryl content of glycinin (Hoshi and Yamauchi, 1983) and freeze drying may result in

irreversible insolubilization of some of the protein (Fukushima, 1980). The free sulfhydryl decrease would presumably have an effect on the tertiary and quaternary structures of the protein and possibly alter antibody binding. Any disulfide mediated protein aggregation may also decrease the soluble protein and decrease the amount of glycinin that is resolubilized, producing a low glycinin content. Thus, it is difficult to ascertain whether the particular soybean sample examined by the Japanese authors was low in glycinin or their methodology produced the lower value.

The discrepancies in the literature concerning soy proteins are illustrated in the different 280nm ultraviolet absorptions of 1 % solutions of the soy proteins glycinin and B-conglycinin which have been reported (Table 1). Iwabuchi and Yamauchi (1987) have suggested that the higher absorption values are due to contamination of the storage proteins with other proteins having greater absorptions. The data for absorption come from different soy cultivars and often the soy variety is not reported. The known genetic variations in subunit composition may have some role in the reported differences but this would probably only be significant if subunits which change in concentration have very high molar absorptions. The possibility of pigmented materials increasing absorption values must also be taken into account in semi-purified samples. It is interesting that in 1987 two different groups reported values for absorption for glycinin and B-conglycinin that differed by 8.0 and 4.6% respectively (O'Neill and Kinsella, 1987b; Iwabuchi and Yamauchi, 1987).

Table 1. Reported ultraviolet absorption values of 1% solutions of glycinin and B-conglycinin

Reference	Absorption[a]	
	Glycinin	B-conglycinin
Badley et al., 1975	8.1	-[b]
Catsimpoolas and Meyer, 1968	9.2	-
Yamagishi et al., 1982	8.04	-
Rao and Rao, 1977	7.9	5.8
Yamauchi et al., 1985	8.04	5.47
O'Neill and Kinsella, 1987b	8.04	4.40
Iwabuchi and Yamauchi, 1987	7.40	4.22
Iwabuchi and Shibasaki, 1981	-	5.47
Yamagishi et al., 1983	-	5.74
Thanh and Shibasaki, 1978	-	4.16
Koshiyama, 1968	-	5.47

[a]280 nm, 1cm, 1% protein solutions.
[b]not reported.

Recently Guzman and Murphy (1988) have shown that highly pure soy proteins in aqueous solutions in the absence of reducing agents have different thermal denaturation patterns than those which have been reported for semipurified proteins in the presence of 2-mercaptoethanol. This illustrates that the choice of model system can have a great effect on the properties observed for the proteins. The study of flavor compound interactions with soy proteins has not been carried out with pure protein solutions in the absence of reducing agents. The proteins have been usually lyophilized before storage, a procedure which, as mentioned, is not without effect on the protein.

Origin of Flavor Problems in Soy

A number of different off-flavor problems including bitter, sweet, green-grassy, burnt, and catty have been described in soy (Schutte and Van den Ouweland, 1979; Cowan et al., 1973; Maga, 1973; Rackis et al., 1979; Sessa and Rackis, 1977; Van den Ouweland and Schutte, 1978). The beany-green-grassy flavor seems to originate from oxidation of lipids present in soy. High levels of lipoxygenase (EC 1.13.11.12) are found in soybeans (Kitamura, 1984). This enzyme catalyses the formation of a hydroperoxide from unsaturated fatty acids containing a 1,4-pentadiene moiety, including linolenic (18:3 n-3) and linoleic (18:2 n-6) acids. The hydroperoxides decompose to form a variety of chemical compounds including aldehydes, alcohols, ketones, esters and acids. The flavor compounds implicated in the beany off-flavor include isopentanol, n-pentanol, n-hexanol, n-heptanol, ethanal, n-hexanal, 3-cis-hexenal, 2-propanone, 2-pentyl furan, and ethyl vinyl ketone (Fujimaki et al., 1965; Hseih et al., 1982; Wolf and Cowan, 1975; Arai et al., 1967; Mattick and Hand, 1969; Smith and Circle, 1972; Kinsella and Damodaran, 1980). Most of these compounds have very low flavor thresholds; Ericksson et al. (1976) have reported that the mean odor threshold for n-hexanal in water is only 19 parts per billion (ppb) with a range of about 6 to 50 ppb. The odor detection threshold for hexanol ranged from about 1600 to 9500 ppb with a mean value of 4865 ppb. Hexanal is often reported as being found in high concentrations in soy products (del Rosario et al., 1984; Wolf, 1975) and it is an important contributor to the

flavor problem because of the low flavor threshold.. Recently, hexanal has been reported to be the most important carbonyl responsible for off-flavor in oxidized oil (Miller and White, 1988), although other compounds, especially vinyl ketones, are involved (Ullrich and Grosch, 1988). The odor thresholds in air can be as much as 1000 times lower for vinyl ketones than hexanal (Ullrich and Grosch, 1988) and their importance in food off-flavor is well known (Hill and Hammond, 1965).

At least three lipoxygenase isoenzymes, L1, L2, and L3, are found in soy. Soybean mutants devoid of each of the isoenzymes separately have been reported (Kitamura, 1984; Hildebrand and Kito, 1984) and strains deficient in both L1 and L3 also exist (Matoba et al., 1985a). Different carbonyl compounds are produced from linoleic and linolenic acids by L1 compared to L2 and L3 (Grosch and Laskawy, 1975). The L1 enzyme, with a optimum pH of 9.0 compared to 6.5 for the other two enzymes, produces significantly lower amounts of carbonyl compounds (Grosch and Laskawy, 1975; Hildebrand and Kito, 1984). Matoba et al. (1985a) report that L2 is responsible for producing n-hexanal when free linolenic acid is used as the substrate. Matoba et al. (1985b) reported that a hydroperoxide lyase (HPL), present in soybeans, catalyzes the breakdown of hydroperoxides to form hexanal. This HPL was found to catalyze the rate-limiting step in the formation of hexanal in L2 deficient soybeans. Davies et al. (1987) examined soybean mutants lacking lipoxygenase isoenzymes. Taste panels found that strains devoid of L2 had higher scores for cereal and dairy

flavors, and lower scores for oily, beany, and rancid flavors. Thiobarbituric acid values (TBA) were lower in L2 null mutants. Soy strains devoid of the other isoenzymes did not have improved scores.

The use of lipoxygenase deficient soybean mutants may decrease the off-flavor problems in soy in the future. However, for any new strains of soybeans to be accepted by farmers, the economic considerations must favor the introduction of these new strains. Because of the unsaturation of soy lipid, it will be necessary to prevent oxidation during processing of soybeans, even in the absence of lipoxygenase.

Protein Flavor Compound Interactions

The number of different possible ways for a ligand to interact with a protein are not great. The binding may be one of a number of types of chemical bonds depending upon the ligand used and the state of the model system (aqueous or dry). Obviously, an alkane can only interact through van der Waals forces or through the hydrophobic effect. Hydrophobic bonding would not take place in a dry model system. Other possibilities for bonding are electrostatic, hydrogen bonding and covalent bonding such as Schiff base. There may also be a combination of more than one type of bonding from one molecule if the nature of the ligand permits it. The different types of bonds have different bonding energies: covalent C-C or C-N, 80-100; covalent S-S, 50; ionic, 5-7; hydrogen, 2-10; hydrophobic (25C), 3-5; van der Waals, 1-2 Kcal/mol (Voilley, 1988).

It is known that the tertiary structure of proteins in aqueous

solution is often stabilized by the hydrophobic bonding of nonpolar sidechains which become buried in the center of the protein molecule (Kauzmann, 1959). In globular proteins, such as the soy globulins, the packing of the amino acids in the center of the protein molecule is very tight, approaching the packing observed in crystallized proteins (Privalov, 1979). During protein denaturation, which involves unfolding of the amino acid chains and structural reorganization, more of the nonpolar amino acid side chains may become accessible to small molecules such as carbonyl compounds (Solms et al., 1973).

There are a variety of techniques which have been used to study the interactions between small compounds and proteins in aqueous solution. These include liquid-liquid partition, gel filtration, equilibrium dialysis, headspace concentration, spectroscopy, and nuclear magnetic resonance (NMR). The first four techniques attempt to determine the amount of bound and free ligand whereas the latter two rely on differences which may occur in the observed parameters as a result of binding. A very useful review of the use of various techniques for determination of flavor protein interactions has been presented by Wilson (1986). Each of the methods have advantages and disadvantages. Liquid-liquid partition involves a biphasic system with the ligand in an organic solvent layered over an aqueous solution of the protein. The system is equilibrated and the amount of ligand in the organic phase is compared for buffered protein and buffer alone. There may be protein denaturation at the interface and the

organic solvent may have some role in the ligand binding. For the gel filtration method, a solution of protein and ligand is equilibrated; protein with bound ligand is separated from free ligand by gel permeation chromatography. The protein ligand equilibration is disrupted during filtration where bound ligand may be disassociated from the protein. Once equilibrium is disrupted, the determined equilibrium binding constant may be affected. Equilibrium dialysis involves a protein solution and solution devoid of protein of opposite sides of a semipermeable membrane. The ligand is added to one of the sides and, after equilibrium, the amount of ligand free and bound is determined from the difference between the two sides. The numerous problems which occur with this technique have been summarized by Wilson (1986). If the ligand is charged, the Donnan equilibrium must be taken into account. Headspace technique relies on the fact that the amount of a ligand in the headspace is directly related to the activity in the aqueous solution by Raoult's Law. The headspace concentrations above solutions of protein and appropriate blanks are compared to determine the amount of bound ligand. The number of compounds which can be examined by this technique and their concentrations are limited by solubility considerations. Nonvolatile compounds cannot be examined. A problem of salting in or salting out of the ligand may arise if the buffer concentrations in the protein solution and blank are not exactly the same. The problems associated with the concentration activity relationship occur in both headspace and equilibrium dialysis. The NMR and spectroscopic techniques are

only valid when the observed changes in relaxation times or line width (NMR) or absorption can be unambiguously interpreted to result from ligand binding.

The interactions between off-flavor compounds and proteins in a dry system has been examined by Wilson and coworkers. The technique employed was gas chromatography using columns of dry soy protein. The affinity of binding is related to the degree of retention by the soy columns, which is determined by retention time differences between a nonadsorbed gas and the compound being studied. This approach has been previously employed for lactose (McMullin et al., 1975). The results for soy protein in the dry system showed that alcohols were retained to a greater extent than aldehydes, acids, and ketones which in turn were retained to a greater extent than alkanes (Aspelund and Wilson, 1983). The authors used the determined thermodynamic free energy of binding to postulate the type of binding which occurred. The alkanes reacted through van der Waals forces alone. In addition to van der Waals forces, alcohols were postulated to form two hydrogen bonds and aldehydes, acids, and ketones formed one hydrogen bond. Crowther et al. (1980) examined the effects of processing on the adsorption of flavor compounds on soy protein. Sheared soy isolate exhibited decreased binding as compared to nonsheared. There was a significant processing temperature-moisture interaction. It was explained that processing decreased the amount of polar binding sites available.

The interactions of alkanes with proteins has been examined by a

number of authors. Mohammedzadeh-K. et.al. (1967) studied the interactions of pentane, heptane, nonane, and mixtures of pentane and nonanone with the proteins bovine serum albumin (BSA), B-lactoglobulin, ovomucoid and ovalbumin. The authors used a partition technique with an alkane layer on top of the buffered aqueous protein solution. After equilibration, 1ml of the aqueous phase was removed and the alkane extracted by using 0.1 ml undecane. This would produce an estimate of the maximum number of binding sites on the proteins because saturation would likely be attained. The time to reach equilibrium was about 8 hours for pentane whereas heptane and nonane required 4 and 2 hours, respectively. There was a greater binding of the smaller compounds with estimated maximum moles alkane bound per mole BSA being 3.8, 2.8, and 1.4 for pentane, heptane and nonane, respectively. The competitive interaction between pentane and nonane seemed to show a slight preference for pentane but the pattern seemed complex. The interaction between these two alkanes should have illustrated differences in affinities if they existed. The four proteins studied showed similar binding of alkanes when expressed on a milligram per gram protein basis with the exception of ovalbumin, which bound approximately half as much as the others. Mangino et al. (1985) examined the application of this method for the prediction of the functionality of whey protein concentrates. They also found that the estimated maximum number of binding sites was greatest for pentane and least for nonane. The correlation between different measures of protein functionality and alkane binding were found to be better than

either the surface or exposed hydrophobicity as determined with the cis-paranaric acid method of Kato and Nakai (1980) which was modified from Sklar et al. (1977). Mangino et al. (1985) hypothesized that as alkanes increase in size, the hydrophobic site necessary for binding would be larger and, from their data, fewer in number. The denaturing agents, urea and dithiothreol, increased the binding of heptane. It should be noted that these authors found that up to 0.316 g ligand was bound per gram protein.

Arai et al. (1970) examined the interaction of hexanal and hexanol with soy protein by using a gel filtration technique. The binding constants obtained were 173.4 and 80.3 M^{-1} for hexanal and hexanol, respectively. Using an estimated molecular weight of 220 KDaltons, about 1.7 sites per mole were present on the soy protein studied.

Nawar (1971) examined the headspace concentrations of a number of ketones, present at concentrations of 75 and 750 ppm, above different solutions including 10% gelatin. The ratio of headspace concentration of control (no protein) and gelatin, which is an estimate of the degree of binding, was greater for 2-pentanone than 2-heptanone, but statistics were not performed.

The interactions of different food components with flavors was discussed by Solms et al. (1973). Data on binding of butanal and 2-butanone to a soy protein isolate were calculated using an estimated protein molecular weight of 50 KDaltons; some of the data were repeated in Beyeler and Solms (1974). The molal binding decreased for

2-butanone and butanal as the protein concentration increased. This might be due to increased equilibrium time with more protein or problems with high protein concentrations of protein blocking the pores of the dialysis membrane. It may also be due to protein-protein interaction at higher concentrations. The binding curves were complex and the authors stated that a stepwise process might occur. The authors suggested that the binding of ligands to proteins involved protein unfolding due to ligand binding to the hydrophobic regions, with the creation of new nonpolar binding sites. The maximum molal binding from the presented curves seemed to be about 80 and 270 for butanal and 2-butanone, calculated by using a estimated molecular weight of 220 KDaltons. The interaction between soy protein isolate and flavor compounds was investigated by using a equilibrium dialysis technique (Beyeler and Solms, 1974). The range of flavor concentrations studied was 10-80,000 ppm. The binding curves were found to be linear (but the same data seem to have been presented by Solms et al. (1973) and was stated to be complex in the earlier publication). The binding constants obtained indicated that butanal was bound with greater affinity than 2-butanone, whereas butanoic acid was found to have no affinity for the protein. The binding constants were reasonably independent of temperature, but statistics were not performed. The authors concluded that binding was likely a combination of electrostatic and hydrophobic forces but the lack of interaction by carboxylic acids was not explained. The methodology of Beyeler and Solms has been questioned by Kinsella and Damodaran (1980)

and Damodaran and Kinsella (1981a). The method used by Beyeler and Solms would not account for nonspecific binding of the ligands to the membrane and the maximum moles bound per mole soy protein was about 1200, from the binding curve. Although the molecular weight used for soy protein was only 50 KDaltons, a realistic estimation of 220 KDaltons would still produce molal binding of around 270 for 2-butanone, a value which seems to be high.

Franzen and Kinsella (1974) used an approach similar to Nawar (1971) to examine the interaction of a series of aldehydes and ketones at a level of 200 ppm with a number of different proteins. Most, but not all, of the headspace concentrations were lower in the presence of soy protein isolate and there did not seem to be a trend for longer chain carbonyls to be bound to a greater extent than shorter chain, in the aqueous model system.

Gremli (1974) looked at the retention of a series of carbonyl flavor compounds by a 5% aqueous solution of soy protein. Headspace concentration was compared with a high-vacuum transfer which, according to the author, represent total and irreversible retention, respectively. The reversible retention of aldehydes increased as the chain length increased and unsaturated aldehydes were retained to a greater extent than the corresponding saturated compounds. The retention of ketones increased as chain length increased up to C10, whereafter the retention decreased. Unsaturated ketones were retained to a lesser extent than the corresponding saturates. Aldehydes were retained to a greater extent than corresponding ketones. Alcohols

were found to have no affinity for the protein. The retention after high-vacuum transfer was greater as aldehyde chain length increased, however, ketones were not retained after vacuum transfer. The data on retention of 2-nonanone and heptanal were given in terms of mg ligand bound per 100ml 5% protein solution, but can be converted to mole bound per mole protein, assuming an average molecular weight of 220 KDalton (Table 2). It can be seen that, at a given ligand concentration, more moles of the aldehyde than ketone were bound per mole protein. The binding curves show no hint of inflection or saturation, indicating that the number of binding sites is likely greater than twice the number of moles bound at the highest ligand concentration employed (i.e., >27 for heptanal and >7 for 2-nonanone). Although the vacuum transfer method is not specific for covalent binding (Schiff base), it illustrates that differences exist between the binding of aldehydes and ketones. The finding that unsaturated aldehydes are bound to a greater extent than the corresponding saturated compounds is of importance because hexenal is an important contributor to the beany off-flavor. The greater retention of unsaturated compounds compared to saturated is difficult to reconcile with the hypothesis that the binding is simply hydrophobic. It is, however, possible that the double bond could cause a bending in the molecule which could increase or decrease the interaction between the ligand and the nonpolar areas of the protein. Gremli did not describe the conformation of the double bonds in the unsaturated compounds.

Table 2. Binding of volatile carbonyls by soy protein isolate calculated from data presented by Gremli (1974)

Ligand	Amount ligand (ppm)	Mole bound/mole protein
heptanal	12.3	0.27
	24.7	0.54
	30.8	1.35
	61.6	2.69
	308.2	13.46
2-nonanone	24.4	0.31
	60.9	0.92
	121.8	1.85
	182.8	2.77
	243.7	3.69

The interaction between a denatured soy protein isolate and benzyl alcohol was examined by King and Solms (1979). The method employed used ^{14}C-benzyl alcohol. After ligand reaction with the protein, the total radioactivity was determined (bound + free) and, after centrifugation, the supernatant gave the free ligand. The binding curve was linear over the concentration range studied. The binding was found to be reversible as almost quantitative recovery of ligand was found after resuspending the protein, precipitated by centrifugation. The amount of adsorbed benzyl alcohol decreased when the model system contained lipid and soluble protein.

Damodaran and Kinsella (1980) examined the interactions of carbonyl compounds with bovine serum albumin. The technique employed was liquid-liquid partition with a sampling tube. The saturation curves presented showed a complex binding pattern with saturation not being attained or even approached. Clear curvature in the Scatchard

plots was observed for 2-nonanone, nonanal, and 2-heptanone. This curvature was explained as suggesting two types of binding sites with different affinities. The number of sites in the first class was extrapolated from the initial slope of the curve. These interesting observations deserve comment. It is well known that the type of curvature which was observed can be explained as either two or more types of sites with differing affinities or as a negatively cooperating single set of sites (Klotz and Hunston, 1979; Klotz, 1986). Klotz (1986) points out that these two possibilities can not be differentiated from binding data alone. The type of extrapolation used by Damodaran and Kinsella (1980) has been severely criticized by Klotz (1982, 1986; Klotz and Hunston, 1979) as "bending thought to wish". Extrapolating an uncertain curve to a point which seems reasonable from the literature is thus a dubious adventure, although it is very satisfying and apparently hard to resist. Damodaran and Kinsella found that the UV absorption difference (between protein alone and protein with various amounts of bound ligand) spectra and fluorescence spectra (280 ex; 250-450 em) changed upon ligand binding. This suggests structural rearrangement, which could cause a negative cooperativity in binding of ligand to one set of binding sites. The authors suggested that the ligand binding by one class of sites caused structural conformation changes which created additional binding sites with a weaker affinity. If this is true, from their data, the conformational changes are underway even as soon as 1 mole ligand is bound per mole protein, because the UV absorption difference and

fluorescence measurements were different even at the lowest levels of bound ligand examined. It is also possible that the conformational changes decrease the strength of the binding site while increasing their number. This would be what one would expect in the highly hydrophobic interior of a protein becomes unfolded because of binding.

Damodaran and Kinsella (1981a) investigated the binding of a series of ketones and nonanal to whole soy protein. The technique employed was equilibrium dialysis. Ligand was extracted into isooctane from both sides of the dialysis membrane. The partition coefficient obtained from aqueous solutions containing no protein were reported to be about 10^{-3}. The binding was said to be reversible and all of the bound ligand extracted into the isooctane, but no evidence for this was given except the statement that a second extraction contained no ligand. A second extraction (with equal aqueous and organic volumes, as was used) of a ligand with a partition coefficient of 10^{-3} is expected by theory to contain 0.1% of the original extracted amount. The binding was said to be simply hydrophobic with no effect of ligand functional group except to inhibit the hydrophobic association. Evidence presented for this was the increase in binding constant as chain length increased in a homologous series of ketones. The binding curves which were presented show no hint of inflection and thus according to Klotz (1982) indicates that the number of binding sites equals, at least, double the highest number of ligands bound per molecule protein. This indicates that the number of binding sites are greater than 4, 7 and 10 for 2-nonanone, 2-octanone and 2-heptanone,

respectively. However, the authors suggested that there were four to five binding sites per molecule from the double reciprocal transformation. It is unfortunate that the authors did not comment on the lack of saturation observed in the binding curves. The binding curves clearly indicate that there are more than four to five binding sites and the lack of saturation make data obtained from the transformations of questionable validity. The binding constant for 2-nonanone was greater at 5 than at 25 and 45C; 2000 compared to 930 for the higher temperatures. Although the authors reported that there was no difference in binding between 25 and 45C, the binding curves for these data suggest otherwise. Thissen (1982) used the data presented in the graphs to calculate the binding constants to be 476 and 833 M^{-1} at 25 and 45C, respectively, although the precision of obtaining data from the presented curves is probably poor. Only the data from 5C seemed to follow the pattern expected if there is only one type of sites with no interactions (the rectangular hyperbola).

Damodaran and Kinsella (1981b) examined the interaction of semipurified soy proteins, glycinin and B-conglycinin, with 2-nonanone. The binding of the ketone was found to be much lower for glycinin than for B-conglycinin, in fact, glycinin was described as having almost no affinity for the ketone. However, the binding curves presented suggest that the binding by glycinin was increasing as ligand concentration increased, but in more of a sigmoidal rather than typical hyperbolic pattern. This might indicate that there is cooperativity in the binding. Increase in ionic strength of glycinin

solution to 0.5 caused an increase in affinity, and 8 binding sites with a binding constant of 290 M^{-1} were reported. Urea decreased the binding constant but increased the number of sites. This was interpreted in terms of structural rearrangement.

Thissen (1982) used a headspace technique to investigate the binding of a homologous series of aldehydes and ketones, as well as hexanol and hexane, to a soy protein isolate. The method used to calculate the binding constant was reported to have a great effect on the values obtained, although the trends among the values were similar. The differences in K would have a profound influence on the calculated free energy which is calculated directly from the binding constant. The concentrations of flavor compounds studied typically ranged from 20 to 4000 ppm. The binding curves showed very clear positive cooperativity for most compounds. A modified Klotz plot indicated that there were three different cooperating sites for all compounds except 5-nonanone, where there were two. There was no detectable interaction between hexane and the soy protein. The thermodynamic parameters indicated that the entropy was driving the reaction. The results were interpreted as showing that ligand binding caused conformation changes in the protein and that this opened new binding sites. The binding was thought to be hydrophobic but there was a clear influence of the functional group. The number of binding sites for ketones were much higher than reported by Damodaran and Kinsella (1981a,b), ranging from 15 for 5-nonanone to 82 for 2-hexanone. 2-pentanone and 2-heptanone both had 50 sites.

The way that the binding curves were drawn by Thissen from her data was to respect the validity of the points, even though the curves were not the familiar smooth hyperbolic shape. This brings up an important point. If the binding is of a complex nature, whether interacting sites, different classes of sites of differing affinity, or structural changes affecting binding site number or affinity, the binding curves would not display the comfortingly smooth shape one would see from a single noninteracting site. The validity of the data points becomes important. Normal statistical variation would lead to a scattering of points around the binding curve. A curve drawn for these data should have randomly distributed residuals. It may be difficult to differentiate random variability in the data from actual deviations from a smooth curve which are elicited from complex binding. However, the random variability in the points of a binding curve would not be reproducible. It is probably important to calculate confidence intervals about every point to see if a smooth curve would fit the data within experimental variation. The data reported by Thissen (1982) had very small variations and thus the deviations from a smooth curve were real.

Einig (1983) investigated the binding of meat aroma compounds to isolated soy glycinin, B-conglycinin and a whole soy globulin fraction in dry systems using a microreactor with volatile trapping in a low moisture model system. The compounds studied included a series of substituted pyrazines and sulfides. Glycinin was found to bind more of the alkyl substituted pyrazines than B-conglycinin. Glycinin also

bound more 2-acetyl-3-methyl pyrazine and 2-acetyl pyrazine at low protein levels but B-conglycinin was found to retain more 6-methyl-2-2'furyl pyrazine. The number of binding sites for the alkyl pyrazines were determined to be greater for glycinin, ranging from 3.6 for methyl pyrazine to 1.3 for tetramethyl pyrazine compared to B-conglycinin which had 3.3 for methyl pyrazine and 0.8 for tetramethyl pyrazine. The Gibbs free energy of binding was greater for glycinin than B-conglycinin for methyl pyrazine but the opposite was reported for the dimethyl, trimethyl, and tetramethyl pyrazines. The G values ranged from -5.514 Kcal/mole to -6.873 Kcal/mole, and increased for both glycinin and B-conglycinin as the weight of the alkyl substitution increased. The number of binding sites for acetyl pyrazine and acetyl thiopene were 5.6 and 2.6 for B-conglycinin and 3.9 and 8.8 for glycinin. The G values were greater for glycinin for the pyrazine but greater for B-conglycinin for the thiopene, ranging from -5.710 to -7.237 Kcal/mole. Methional was completely retained by both of the soy proteins; free methional was not observed at the highest ligand level employed. The author concluded that the reaction between meat flavor compounds and soy proteins was through hydrophobic and electrostatic bonds whereas hydrogen bonds were important for the carbonyl compounds, in the low moisture model system used.

Okitani et al. (1986) examined the reaction between hexanal and protein. Exposure to vaporized hexanal at 75% humidity caused impairment of lysine, tryptophan and methionine residues. The reaction product with lysine and the aldehyde was a substituted

pyridinium betaine. The mechanism of the reaction presumably follows the Schiff base aldol condensation well known as initial steps in Maillard browning. Oxygen was found to be necessary as an oxidizing agent for formation of the pyridinium ring. Methionine groups were converted to methionine sulfoxide residues. This latter fact is important as sulfur amino acids are the limiting amino acids in soy.

The binding of diacetyl by pea proteins was examined by Dumont and Land (1986) using a headspace technique. Isoelectric precipitation of the protein decreased the ligand binding and some previously bound ligand was released. The affinity of diacetyl for legumin was higher than for vicillin. On a basis of 220 KDalton molecular weight, about 18 sites were present in a whole protein isolate. The binding was hypothesized to take place between arginine residues in the protein and the ligand.

The interactions between chicken flavoring and added soy protein in a formulated soup were studied by Malcolmson et al. (1987). Taste panels evaluated soy flavor, chicken flavor, overall flavor and pleasantness, by using magnitude estimation. The experimental design was a 4X4 factorial but only the results of a Tukey's multiple comparison were given; the significance of the main effects and the interaction were not reported. Added soy protein up to 16% had no effect on the chicken flavor. The pleasantness of the soup formulations was lower at 16% soy than lower levels of added soy at 0.4, 0.2 and 0.0% chicken flavoring. The authors suggested that this was due to an undesirable soy flavor perceived at this level, but

their data suggested that the perceived soy flavor was not significantly different at 8 and 16% soy, although a linear trend seemed clear. It is difficult to interpret data where a partial statistical analysis is reported, and a thorough treatment including effects, linear components of these effects, and interactions are omitted.

O'Neill and Kinsella (1987a) have examined the interaction between B-lactoglobulin (B-LG) and a homologous series of ketones. The binding constants for a were 150, 480, and 2400 M^{-1} for 2-heptanone, 2-octanone, and 2-nonanone, respectively, and one binding site was found in the B-LG monamer. The change in free energy was 700-1000 cal/CH_2 residue. Urea decreased the binding affinity but not the number of binding sites. The authors suggested that urea caused partial unfolding which did not destroy the hydrophobic site but reduced the hydrophobic binding region.

O'Neill and Kinsella (1987b) examined the binding of 2-nonanone to purified soy protein fractions. SDS-PAGE indicated that the B-conglycinin fraction was about 90% pure whereas only minor contaminants were detectable in the glycinin. The range of free flavor concentration studied was about 1 to 10 $x10^{-4}$ M for whole soy and about 1.7 to 10 x 10^{-4} M for B-conglycinin and glycinin; corresponding to about 15 to 173 ppm and 28 to 173 ppm, respectively. Gremli (1974) reported that saturation of a soy protein isolate was not achieved at total levels of 244 and 308 ppm, for nonanone and heptanal. O'Neill and Kinsella (1987b) reported the number of binding

sites to be 2.8 for B-conglycinin and 9.9 for glycinin. The binding constant was about $3136M^{-1}$ for B-conglycinin and $539M^{-1}$ for glycinin. These are strikingly different from constants provided by the same laboratory earlier (Damodaran and Kinsella, 1981a,b). These data were calculated using the correct molecular weights and not the molecular weight of 100 KDalton which was employed by Damodaran and Kinsella (1981a,b). The binding curves were not illustrated for whole soy or B-conglycinin, but the curves for glycinin and the glycinin acidic and basic subunits showed no hint of inflection. This indicates that saturation was not approached and only a very limited range of the saturation curve was actually investigated. The same equilibrium dialysis technique as Damodaran and Kinsella (1981a,b) was employed and as in their previous work the buffer contained 2-mercaptoethanol and sodium azide. The effect of these compounds on ligand binding has not been reported. The protein preparations used by Kinsella and coworkers have been freeze-dried.

Evidence for irreversible binding

The measure of irreversible binding described by Gremli (1974) is likely an overestimation. The difference between true covalent irreversible binding and ligand binding which is not reversible by high vacuum treatment or solvent extraction is not known. The fact that irreversible binding, as determined by Gremli, increased as chain length increased could be due to hydrophobic bonding which is not irreversible or to the higher boiling points of the longer chain carbonyls, making their removal by vacuum treatment more difficult.

Okitani et al. (1986) found Schiff base formation between proteins and hexanal in a model system at 75% humidity. It is likely that this reaction will take place in aqueous systems or when heat treatment is involved such as desolventization of defatted soy flakes in industry.

Prevention or Removal of Off Flavor Compounds

High temperature prevention

Wolf (1975) describes a number of methods of decreasing the off-flavor problem which utilize heat treatment to deactivate the lipoxygenase: 1. grinding with hot water; 2. dry heating of whole beans; 3. soaking, then blanching (Illinois process); 4. grinding at low pH prior to cooking; 5. alcohol treatment. Borhan and Snyder (1979) utilized ethanol soaking and heating of the whole beans to destroy lipoxygenase while maintaining NSI (nitrogen solubility index) at 40-50. The NSI is essentially the percent of the total nitrogen that is soluble. Maintaining the NSI as high as possible is necessary when the soy proteins are destined for use for their functional properties. The previous techniques may produce acceptable bland proteins, nevertheless protein functionality is impaired.

Antioxidant infusion

Kinsella and Damodaran (1980), in a review on the soy protein problem, suggest that, although antioxidant infusion or addition of antioxidant before milling have been reported, this approach is likely too expensive to be practical.

Enzymatic removal of flavor compounds

The specificity and catalytic efficiency of enzymes make enzyme application to the removal of flavor compounds attractive. Takahashi et al. (1979a) examined the use of bovine liver aldehyde dehydrogenases (ADH) which oxidize aldehydes to carboxylic acids. The sensory threshold values of aldehydes are much lower than alcohols or carboxylic acids. Conversion of protein bound aldehydes to corresponding acids should, thus, reduce off-flavor intensity. Alcohol dehydrogenases (ALDH) convert aldehydes to alcohols, but small amounts of aldehydes may still be present even after the reaction and alcohols themselves contribute to the off-flavor problem. The essentially irreversible oxidation of aldehydes to acids is therefore preferred. Takahashi et al. (1979a) isolated three distinct aldehyde dehydrogenases from bovine liver: cytosolic, mitochondrial and microsomal. The mitochondrial enzyme was found to have a broad specificity for straight-chain saturated aldehydes (C2 to C10) and had lower rates with unsaturated aldehydes. The Km for the mitochondrial enzyme was independent of chain length whereas the Km was higher with shorter aldehydes for cytosolic and microsomal enzymes. For this reason, the mitochondrial enzyme was considered superior for application to the off-flavor problem. The lower the Km the more effective the enzyme will be at low concentrations of substrate. Characterization of the mitochondrial enzyme showed that the pH optimum for activity was 9.3 and the enzyme was stable from pH 6 to 8 with substrate present. The temperature for maximum activity was 50

C. The enzyme was found to have poor thermostability; 15 minutes at 30 C destroyed 30 % of the activity with substrate present.

Chiba et al. (1979a) examined the application of bovine liver ADH to soymilk and soybean extracts. It was found that there was endogenous ALDH activity in a defatted soy extract that was thermolabile. The endogenous enzyme converted alcohols to aldehydes that could then be oxidized to acids by using ADH. Off-flavors were removed from the soy products studied using the enzyme application and the off-flavors did not return on storage. Acid precipitation of the soy proteins increased the off-flavor binding but pepsin hydrolysis did not. Sasaki et al. (1981) found that an aldehyde eliminating activity of unheated soy was dependent upon pH and flour-to-water ratio.

A problem with the possible use of bovine mitochondrial ADH is that a nicotinamide cofactor is necessary as an electron acceptor. To circumvent this problem, Takahashi et al. (1979b) examined the use of bovine liver aldehyde oxidase (AOX). This enzyme uses O_2 as an electron receptor and produces H_2O_2. The enzyme was reported to be unstable at neutral or acidic pH. The Km increased with increasing chain length for straight-chain saturated aldehydes. Catalase could be used in a coupled system to catalyze the conversion of the H_2O_2 to H_2O and O_2. The AOX was found to be inferior to the ADH at oxidizing bound aldehydes. Because of this, the ADH was further examined and diaphorase was reported by Takahashi et al. (1980) to be effective in regenerating NADH from NAD^+. This could decrease the cost involved in

using large quantities of the expensive nicotinamide cofactors.

The ability of enzymes to remove off flavor compounds from soy proteins indicates that the flavor compounds are free to react with the enzyme. The removal of the free flavor compounds from the solution would, from the equilibrium, cause dissociation of bound ligands, which may then be converted to products by the enzyme. It is unlikely that the enzyme would act on ligands bound to nonpolar sites in the protein structure because of steric hindrance but Chiba et al. (1979b) reported that ALDH oxidized protein bound aldehydes.

Microbial fermentations

Fermentation of soymilk by Lactobacillus species has been shown to reduce beany flavors (Wang et al., 1974). Friend et al. (1987) have patented a process for reducing the off-flavors in vegetable protein solutions by using contact with Rhizopus or Aspergillus molds under controlled conditions. The mechanism of the off-flavor reduction was presumably via enzymes such as ALDH. The effect of these treatments of the functional and nutritional properties has not been reported but would be an extremely interesting area of future research.

Azeotropic solvent extraction

The off-flavor compounds found in soy products may be reduced or removed by using azeotropic solvent (hexane-alcohol) extractions (Wolf, 1975; Cowan et al., 1973; Rackis et al., 1979). The azeotrope extractions generally reduced the solubility of the protein. Kinsella and Damodaran (1980) have pointed out that the cost of the process may

make it impractical and the solvents or thermal treatment used to remove the solvents would cause unacceptable protein denaturation. The use of azeotropic solvent in the initial lipid extraction, in the place of nonpolar solvent, may remove the flavor compounds from the protein, but the flavors would become a problem in the oil, however, deodorization would likely remove the lower boiling off flavor compounds from the oil.

Techniques for Determination of Binding Parameters

The basic methods which can be employed for the analysis of binding data include transformation of the binding curve and more complicated stoichiometric and site approaches (Klotz, 1986; Klotz and Hunston, 1975, 1979; Connors, 1987). The site, also known as microscopic, approach attempts to obtain information about individual binding sites whereas the stoichiometric technique looks at sequential binding. A computer program using a site approach was reported by Fletcher and Spector (1968). The site approach suffers from the limitations that it becomes unmanageable if site affinity changes with occupancy, may not be applicable if all binding sites do not exist initially, and the constants which are obtained may have no simple correspondence to the site binding affinities (Klotz and Hunston, 1979). The constants obtained from the stoichiometric approach do not provide information on the binding affinities of the individual sites and may be difficult to interpret (Connors, 1987). The stoichiometric approach was reported to be the more versatile format for the thermodynamic analysis of ligand-protein interactions (Klotz and

Hunston, 1975) and a computerized stoichiometric approach has been described (Fletcher et al., 1970). The third method is to examine the data with techniques developed for the analysis of simple binding systems; one binding class of sites with no interaction. This would include the Scatchard and Klotz approaches. A modification of the Klotz plot for use in positive cooperating systems has been reported (Segal, 1975). The third approach has been used by all researchers examining the aqueous interactions of flavor compounds with soy proteins except Thissen (1982) who also used the computerized stoichiometric method of Fletcher et al. (1970). However, the stoichiometric method did not provide a binding model which fit the data obtained by Thissen. It was thought that the program, as written, did not handle the drastic changes in slope correctly and cancelled them out.

The simple binding of ligand to protein is described by the equation: $V=nKA/(1+KA)$

where V is moles ligand bound per mole protein

n is the number of binding sites per protein molecule

K is the equilibrium binding constant

A is the molar concentration of free ligand at equilibrium.

The graph of this equation is a rectangular hyperbola with the maximum value of the Y-axis corresponding to the number of binding sites and the point on the X-axis which corresponds to Y-max/2 equaling the binding constant. A plot of V against log A produces a binding curve which has an inflection at Vmax/2. The equation can be

transformed via a number of procedures to produce straight lines and simplify determination of Vmax and K. However, the use of the transformations for limited amounts of data where saturation is not approached has been criticized (Klotz, 1982; Connors, 1987). The transformations have taken the names of certain authors who have reported their use in different domains (Klotz, 1986). It is not uncommon to see the same transformation with three different common names. Connors (1987) uses an easy descriptive terminology. The double reciprocal plot, also known as Klotz, Hughes-Klotz, Lineweaver-Burk, Benesi-Hildebrand etc., is formed by taking the reciprocal of both sides of the hyperbolic binding equation. The equation thus becomes: $1/V=1/n+1/(nAK)$. A plot of the reciprocal of V versus the reciprocal of A gives a straight line with a slope of $1/Kn$ and a y-intercept of $1/n$. Another common transformation is the X-reciprocal, also known as Scatchard or Eadie (Connors, 1987), which is formed by dividing both sides of the equation by A. This equation takes the form: $V/A=nK-VK$. This plot is not open-ended and has both X and Y intercepts. A plot of V/A against V produces a straight line with slope -K and an X intercept of n. A third transformation which is used is the Y-reciprocal plot. This equation has the form: $A/V=A/n+1/nK$. A plot of A/V versus A gives a line with a slope of $1/n$ and a X intercept of -K.

The binding data may be clearly nonlinear after transformation. This is indicative of cooperative binding, a number of different classes of binding sites, or numbers of binding sites and their

affinities changing. Negative cooperativity and several classes of binding sites cannot be differentiated from the curves alone (Klotz, 1986). Positive cooperativity can be determined from the curve and the modification described by Segal (1975) allows the overall binding constant to be determined as well as the number of different sets of binding sites and total binding sites. The modified equation takes the form: $1/V=1/n+1/(nA^{Y}K)$. Y in this equation determines the minimum number of different binding constants. This approach assumes that the cooperativity is high. For this reason, the number of different binding constants as determined from this equation should be considered an apparent number; if the cooperativity is low, the apparent Y would be less than the actual value.

A problem arises: which type of plot is best. Each transformation has its proponents and critics. The biggest problem with the double reciprocal plot is that, when values as A are chosen to be equally spaced, the points are concentrated at low values of 1/A because of the transformation. This imposes problems with least-squares linear regression because the values at low levels of A, high levels of 1/A, tend to be emphasized without weighting. However, this transformation does have the advantage that the variables V and A are kept separate whereas the other transformations mix these two variables. This is important as far as the statistical analysis is concerned because the uncertainty in V is usually greater than A. The least squares regression is constrained to have a minimum, even distribution of error in the independent variable (Connors, 1987).

The weighting factors necessary for the transformations are more constant for the X-reciprocal plot than double reciprocal (Wilkinson, 1961). All of the linearizing transformations suffer from the problem of distortion of experimental errors (Motulski and Ransnas, 1987).

Wilkinson (1961) addressed the problem of data analysis and presented weighted and nonlinear regression methods. This technique uses a linear regression to produce initial estimates which are refined with nonlinear methods. This seems to be a very good approach which avoids the problems with transformations.

Connors (1987) points out that, for the equilibrium constant to be reliably interpreted, more than half of the range of the saturation curve must be examined. Klotz has discussed the problems associated with only examining part of the binding curve and the problems in determining the value of n from transformations of partial curves. In theory, a complete saturation curve is best because it unambiguously gives useful estimates of n and K from the binding curve, and the tranformations in this case would be expected to provide reliable data. Unfortunately, it is commonly difficult to obtain data over all of the binding curve. It is apparently important to follow the binding, at least, up to the inflection point of the semilog binding curve (Klotz, 1982).

Thermodynamic Parameters

The free energy of the reaction of a ligand with a protein can be obtained from the equilibrium binding constant from the following equation: $\Delta G=-RT\ln K$ where Δ indicates change in the parameter, G is

the Gibbs free energy of interaction, R is the gas constant (1.987 cal $deg^{-1}mol^{-1}$), T is the absolute temperature ^{o}K, and K is the binding constant (Wiesinger and Hinz, 1986; Kinsella and Damodaran, 1980). The enthalpy change can be determined from the equilibrium binding constants determined at two temperatures: $\Delta H = (-R \ln K2/K1)/(1/T2-1/T1)$. The change in entropy is determined from the relationship: $\Delta S = (\Delta H-\Delta G)/T$.

These thermodynamic parameters are not a magic formula to determine the nature of binding, but the binding must be consistent with the thermodynamics of the reaction. For example, hydrophobic bonds induce a decreased structuring of the water in the vicinity of the nonpolar solutes, which causes an increase in entropy (Kauzmann, 1959). Although electrostatic bonds are also driven by entropy, the two can be differentiated because electrolyte addition would strengthen hydrophobic but weaken electrostatic bonds.

Determination of Protein Hydrophobicity

The surface polarity of proteins seems to be related to certain functional properties of these proteins. Nakai (1983) describes a number of possible techniques to determine the apparent protein hydrophobicity: 1. reverse-phase chromatography; 2. hydrocarbon binding; 3. hydrophobic partition; 4. fluorescent probe and; 5. salting out-surface tension. Turner and Brand (1968) report the use of N-acrylaminopthalenesulfonates (ANS) as fluorescent probes to determine the polarity of protein binding sites. Although the mechanism involved in the polarity-induced fluorescence of the ANS

compounds is not known, the concept that the observed changes in fluorescence are related to the polarity of the environment surrounding the ANS species seems sound. Sklar et al. (1977) found that conjugated fatty acids with partial trans character could be employed as fluorescent probes of hydrophobic sites on proteins. Mangino et al. (1985) have pointed out that the binding of these acids to proteins may involve both nonpolar and electrostatic bonds. Early studies by Nakai examined the use of hydrophobic chromatography and polar-nonpolar phase partition techniques. Kato and Nakai (1980) have examined the use of the method of Sklar et al. (1977) in the examination of protein functionality hydrophobicity relationships. Kato and Nakai (1980) pointed out that there is no convincing reason that, when fluorescent enhancement occurs with ANS binding, the binding must be hydrophobic; but later studies by the same laboratory (Hayakawa and Nakai, 1985) have shown the usefulness of the ANS compounds as hydrophobic probes. The cis-paranaric acid method has been used to determine both surface hydrophobicity and total hydrophobicity (after protein unfolding). The total, but not surface hydrophobicity, has been correlated to protein foaming capacity (Townsend and Nakai, 1983). The surface hydrophobicity correlated well with emulsification capacity (Townsend and Nakai, 1983) but not with the thermal properties of gelation, thickening and coagulation (Voutsinas et al., 1983). Kato et al. (1984) point out that the cis-paranaric acid method is not applicable to insoluble proteins and propose a method involving binding of sodium dodecylsulfate (SDS) at

levels which do not promote protein unfolding. Although the binding of SDS and cis-paranaric acid were well correlated, the change in SDS binding was much less than cis-paranaric acid for a given change in protein polarity, indicating that the cis-paranaric acid method would be much more sensitive. The surface hydrophobicity of crude fractions of the soy proteins glycinin and B-conglycinin were determined by both the cis-parinaric acid and SDS methods (Kato et al., 1984). B-conglycinin was found to have higher surface hydrophobicity by both methods but the difference in the hydrophobicity seems more apparent by the cis-parinaric method (cis-parinaric acid: 7S 247, 11S 96; SDS: 7S 2.6, 11S 2.0). Kato et al. (1984) report that as the ionic strength increased from 0.1 to 1.0, the surface hydrophobicity of soy proteins increased and that surface hydrophobicity increased during heat denaturation. Hayakawa and Nakai (1985) found that the binding sites for ANS and cis-paranaric acid may be different and suggested the terminology aromatic and aliphatic hydrophobic binding sites. ANS hydrophobicity was correlated to protein insolubility but cis-parinaric acid hydrophobicity was not. However suggestive this may be that the binding sites are different for these two hydrophobic probes, clear evidence to this effect has not yet been presented.

SUMMARY AND OBJECTIVES OF THE PRESENT STUDY

The picture, thus, seems far from complete for the binding of flavor compounds to soy proteins. If the binding is simply hydrophobic, as suggested by Kinsella and coworkers, the lack of interaction for alkanes (Thissen, 1982), alcohols (Gremli, 1974), and carboxylic acids (Beyeler and Solms, 1974) is not explained. Gremli (1974) found clear differences between ketone and aldehyde binding that were not dependent of the size of the hydrophobic part of the ligands. The number of binding sites and binding constants presented by Damodaran and Kinsella (1981a,b) were extracted from incomplete binding curves. The number of binding sites determined by Thissen (1982), Beyeler and Solms (1974) and from the data of Gremli (1974) suggests that there are many more than the 4-5 sites suggested by Kinsella. The binding constant for 2-nonanone was higher at 5C° than at higher temperatures (Damodaran and Kinsella, 1981a). The proposed structural rearrangement causing stronger hydrophobic regions goes against the weakening of hydrophobic bonds one would expect at lower temperatures. If the hydrophobic binding sites are in the interior of the soy proteins where the nonpolar residues are concentrated, it does not seem possible that the hydrophobic regions could reorganize to more nonpolar regions because of lower temperatures. The glycinin and B-conglycinin used by Kinsella were semi-pure and other researchers used soluble or insoluble soy isolate, which is a mixture of a number of different proteins, making the use of the simple transformations of the binding curve not applicable, and the results obtained of

questionable use. The use of sodium azide and 2-mercaptoethanol in the buffered protein solutions may produce data which would not be representative of food systems where these compounds would not be present. Their effect on binding has not been investigated. The soy proteins are commonly lyophilized before storage. This has been shown to affect free sulfhydryl groups, which could cause structural changes and affect results obtained. The possibility of covalent Schiff base formation has sofar been ignored by researchers, except in a model system with vaporized hexanal which caused impairment of a number of different amino acid groups (Okitani et al., 1986).

For the reasons outlined above, this study was initiated to investigate the binding of flavor compounds to soy proteins. The study consists of two phases. First, investigation of the binding of hexanal to soy protein. In this phase, the effect of ionic strength, and a reducing agent will be examined. The change in free lysine will be determined. The hydrophobicity of the protein will be estimated by the ANS probe method and the turbidity induced by ligand binding will be measured. The binding of polyclonal antibodies to the protein before and after the reaction with the ligand will be determined. The binding of hexanal will be examined over a sufficient concentration range to achieve saturation if the model system allows it. In the second phase, the binding of a homologous series of ketones, alcohols, aldehydes, and hexane will be examined at several temperatures to determine thermodynamic parameters. This information will be useful for the soy processors interested in removal of the beany flavors.

PART I. DETERMINATION OF THE BINDING OF HEXANAL TO SOY PURIFIED GLYCININ AND B-CONGLYCININ IN AN AQUEOUS MODEL SYSTEM BY USING A HEADSPACE TECHNIQUE

Introduction

It is well established that the use of soy proteins as a functional ingredient in food products would be increased if the beany off-flavor problem is solved (Kinsella and Damodaran, 1980). Off flavor compounds implicated include a number of volatile compounds which are formed via oxidation of the unsaturated lipid present in soy. The compounds have been reported to include pentanol, hexanol, heptanol, hexanal, 3-cis-hexenal, 2-propanone, 2-pentyl furan, and ethyl-vinyl ketone (Hill and Hammond, 1965; Hseih et al., 1982; Arai et al., 1967; Sessa and Rackis, 1977; Rackis et al., 1979; Cowan et al., 1973). These compounds are associated with the proteins in soy and are not completely removed by the techniques employed in the production of soy protein concentrates and isolates. The compounds are carried into the final product and can cause unacceptable flavors. The binding of added flavors by soy proteins can cause problems in determining the appropriate level of flavoring during product formulation.

The association of volatile carbonyl compounds with aqueous solutions of soy proteins has been studied by a number of authors. Arai et al. (1970) have used a gel-filtration technique to examine the binding of hexanol and hexanal to native and denatured soy proteins.

They reported that the binding constant was greater for hexanal than hexanol, but the maximum number of binding sites which can be estimated from their data was about 1.7 for both compounds. As the degree of protein denaturation increased, the retention of these compounds decreased. Gremli (1974) looked at the retention of a series of carbonyl flavor compounds by a 5% solution of soy protein. The retention of aldehydes increased as the chain length increased and unsaturated aldehydes were retained to a greater extent than the corresponding saturated compounds. The retention of ketones increased as chain length increased up to C10 after which the retention decreased. Unsaturated ketones were retained to a lesser extent than the corresponding saturates. Aldehydes were retained to a greater extent than corresponding ketones. Alcohols were found to have no affinity for the protein. The retention after high-vacuum transfer was greater as aldehyde chain length increased, however ketones were not retained after vacuum transfer. The number of binding sites which can be estimated from the data presented by Gremli (O'Keefe, 1988) is >27 and >8 for heptanal and 2-nonanone, respectively.

Solms et al. (1973) and Beyeler and Solms (1974) examined the binding of butanal and 2-butanone, at levels ranging from 10 to 80,000 ppm, to a soy protein isolate. The data were calculated using a molecular weight of 50 KDalton (KDa), which is much lower than either glycinin (320KDa) or B-conglycinin (160KDa), the major storage proteins of soybean. The maximum molal binding which can be estimated from the presented curves, by using a realistic molecular weight

estimate of 220KDa, seems to be 80 and 270 for butanal and 2-butanone. Solms et al. (1973) suggest that the the binding of ligands to hydrophobic regions of soy proteins causes protein unfolding with the creation of new binding sites. Franzen and Kinsella (1974) examined the interactions of aldehydes and ketones with proteins. Most, but not all, of the headspace concentrations were lower in the presence of protein and there was no trend for longer chain carbonyls to be bound to soy protein isolate to a greater extent than shorter chain.

Damodaran and Kinsella (1981a,b) investigated the binding of carbonyls to soy proteins. An increase in the binding constant as chain length increased for 2-ketones was given as evidence that the binding was simply hydrophobic. The binding curves which were presented showed no hint of saturation, indicating that the number of binding sites was at least twice the greatest number observed; that is, higher than 4, 7, and 10 for 2-nonanone, 2-octanone, and 2-heptanone, respectively. However, the authors suggested that there were 4 to 5 binding sites from the double reciprocal (Klotz) transformation. The molecular weight which was used in calculations was 100 KDalton, which is incorrect. The binding constant for 2-nonanone was higher at 5 than 25 and 45C; 2000 compared to 930 M^{-1}. Although the authors reported that the binding at 25 and 45C was similar, the binding curves presented suggest otherwise. Using semipurified proteins obtained from the method of Thanh and Shibasaki (1976), the authors reported that the binding constant for 2-nonanone with B-conglycinin was much greater than glycinin. The binding

curves presented illustrate that glycinin had a sigmoidal rather than typical hyperbolic pattern; the degree of binding was increasing as free ligand increased. This suggests that positively cooperative binding was taking place. Increasing the ionic strength of a glycinin solution to 0.5u caused an increase in affinity, and a binding constant of 290 M^{-1} with 8 binding sites was reported. Urea decreased the binding constant for B-conglycinin but increased the number of binding sites.

Thissen (1982) investigated the use of a headspace technique to determine binding parameters for a series of aldehydes, ketones, hexanol and hexane with a soy protein isolate. The concentrations of the compounds studied ranged from 20 to 4000 ppm. The binding curves indicated that positive cooperativity was present and the modified Klotz (double reciprocal) plot indicated that at least three different cooperating sites were present. There was a clear effect of functional group because hexane was found to have no affinity. The number of binding sites for ketones were higher than reported by Damodaran and Kinsella; 50, 82, 50, and 15 sites were found for C5, C6, C7, and C9 2-ketones, respectively, calculated by using a modified Klotz plot. An increase followed by decrease in number of binding sites could indicate that the size of the binding sites is finite. Mangino et al. (1985) hypothesized that as a ligand exceeded the binding site size, the number of sites become fewer in number. Thissen pointed out that the methods, used in the literature, to calculate the binding constant gave greatly different results; for 2-

hexanone, the binding constants at 25C were 33.3, 1471, 146, and 290 calculated by using the methods of Damodaran and Kinsella (1981a), Beyeler and Solms (1974), Arai et al. (1970), and the modified Klotz procedure, respectively.

O'Neill and Kinsella (1987) examined the binding of 2-nonanone to purified soy glycinin and B-conglycinin. The protein purification involved a combination of the techniques of Thanh and Shibasaki (1976) and Kitamura et al. (1974). The number of binding sites was reported to be 2.8 and 9.9 for glycinin and B-conglycinin using correct molecular weights. The binding constants were 3136 and 539 M^{-1} for B-conglycinin and glycinin, respectively, when correct molecular weights are used. The binding curve illustrated for glycinin indicated that saturation was not approached. The range of moles bound which was examined for glycinin was only 0.3 to 1.0. If the number of binding sites was truly 9.9, the authors only investigated about 10% of the binding curve. Extrapolation of a small fraction of a binding curve to obtain binding parameters has been criticized (Klotz, 1982, 1986; Klotz and Hunston, 1975, 1979; Connors, 1987). The difference in results obtained by Damodaran and Kinsella (1981b) was attributed to the use of incorrect absorptions of a 1% solution at 280 nm in the earlier work.

The proteins used by Kinsella and coworkers were freeze dried for storage. Freeze drying of proteins for has been reported to decrease the sulfhydryl content of glycinin (Hoshi and Yamauchi, 1983) and may result in irreversible insolubilization of some of the protein

(Fukushima, 1980), including B-conglycinin (Hoshi et al., 1982). The effect of these structural changes on ligand binding is unknown.

The binding of carbonyl flavor compounds to soy proteins cannot be easily explained as resulting simply from hydrophobic bonding. The lack of interaction of hexane (Thissen, 1982), carboxylic acid (Beyeler and Solms, 1974), and alcohols (Gremli, 1974) is not explained. The binding parameters obtained by O'Neill and Kinsella (1987) and Damodaran and Kinsella (1981a, b) cannot be trusted because they were obtained by extrapolation of very small portions of the binding curves, the fact that the proteins used in the earlier study were only semi-pure, and the unknown effect 2-mercaptoethanol and sodium azide would have on binding. For these reasons, the binding of hexanal to purified soy proteins was examined.

Materials and Methods

Purification of soy proteins

Soybeans of the variety Vinton 81 were obtained from the Agronomy Department at Iowa State University. The beans were stored in a cold room at 5 C in a tightly closed container until use. The beans were ground in a spice mill and the lipid was extracted by stirring the ground beans with numerous changes of Skelly B (soy:solvent, 1:5, v/v) at room temperature until no further lipid was extracted; usually about ten solvent changes were necessary. The defatted soy was further ground in the spice mill and passed through a #60 mesh sieve to remove hull fragments. A crude protein fraction was prepared by

using the method of Thanh and Shibasaki (1976) with the following modification. In place of two isoelectric precipitations at pH 6.4 and 4.8, three precipitations were done at pHs 6.4, 5.3 and 4.8. The fraction precipitating at 6.4 was crude glycinin, the fraction precipitating at pH 5.3 was discarded, and the fraction precipitating at pH 4.6 was crude B-conglycinin. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed that the crude glycinin fraction was contaminated with B-conglycinin and vice versa. The precipitated fractions were resolubilized in phosphate buffer (45mM phosphate, pH 7.4, 10 mM 2-mercaptoethanol, 0.02% sodium azide, 400mM NaCl). The crude B-conglycinin fraction were purified by passage through Sepharose 6B-Cl twice and the crude glycinin fraction was similarly treated and was further purified by using Concanavalin A-Sepharose 4B (Kitamura et al., 1974). The B-conglycinin was eluted from the Concanavalin A column with the phosphate buffer containing 0.01 M methyl-D-mannoside. Fractions were collected each 30 min (13 ml), protein was determined by UV 280nm absorption and the fractions of interest were concentrated by using ultrafiltration with 50K Nucleopore membranes. The proteins were stored at 5C in concentrated solution (>40 mg/ml) in phosphate buffer containing 0.02% sodium azide and 10 mM 2-mercaptoethanol.

Before use in the binding studies, the proteins were dialyzed at 5C against standard Tris-HCl buffer (pH 8.0, 30mM) in dialysis sacs which were filled one-third full. The dialysis buffer was changed daily for four days. The protein solutions were diluted with standard

Tris buffer to 12mg/ml, placed in new dialysis sacs and the proteins further dialyzed for one day. The protein solutions were diluted to 10.0mg/ml with standard Tris buffer. Protein concentrations were determined by using the Biuret method with BSA standard.

Sodium dodecyl sulfate polyacrylamide electrophoresis

A 10-15 % gradient polyacrylamide gel was prepared by using acrylic cells of equal volume (Laemmli, 1979). The acrylamide to bis ratio was 37.5:1. The resolving and stacking gel pH were 8.8 and 6.8, respectively.

Antibody production and rocket immunoelectrophoresis

The method of Murphy and Resurreccion (1984) was used. Briefly, the purified soy proteins glycinin and B-conglycinin were subjected to preparative SDS-PAGE. The glycinin acidic (including A5) and basic subunits and the a,a' and b subunits were cut from the gels and were eluted into dialysis sacs by using a tube electrophoresis apparatus. This procedure ensured that minor contaminants, which may not be removed by using other techniques, are avoided. The small amount of other proteins could produce a less specific polyclonal response. The purified proteins were used to produce polyclonal antibodies to native glycinin and B-conglycinin. Freund's complete adjuvant was emulsified with about 1 mg of protein by using syringes connected with a small tube, and the solution was injected subcutaneously into the necks of 6 week old male goat kids. Blood was sampled before the first inoculation and at subsequent boosting, where Freund's incomplete adjuvant was used. When a sufficiently high titer was obtained, as

determined by micro Ouchterlony (dried plates were obtained from Sebia, Paris, France), 200-250 ml of blood was withdrawn by using 50ml plastic syringes fitted with 18.5 gauge needles. Several goats were used for each protein because of the variable antigenic response elicited in the goats. The plasma was obtained by ringing the container containing the blood and allowing the clot to form overnight at room temperature. The plasma was further purified by using the method of Mayer and Walker (1980) which involved ammonium sulfate fractionation and overnight dialysis against phosphate buffer at pH 7.4 at 5C. The DEAE-cellulose column was found to be unnecessary. The plasma proteins were precipitated with 75% saturation ammonium sulfate and frozen at -35C until use. Depending upon the titer, from 0.5 to 3.0 ml of plasma was cast into 30 ml of 1% agarose in pH 8.6 Tris-tricine buffer (Weeke, 1979). A standard curve was typically linear from 300 to 5000 ng protein. Wells were cut in the agarose gels and 10.0ul of the protein solutions were added to the wells. Care was taken to ensure rapid protein loading to minimize diffusion. Cross reactivity was tested and was absent.

Syringe calibration

A 1.0ul syringe was obtained from Hamilton (Reno, NV). The calibration of the syringe was determined by using a dye transfer procedure. Briefly, a 40.0 mg/ml solution of bromophenol blue dye in pH 9.9 distilled water was serial diluted from 1:200,000 to 1:100 which represented concentrations of 0.2 to 400ng/ul. The range of 0.2 to 40 ng/ul was determined to follow Beers law (r^2=0.9999). The 1.0ul

syringe was used to transfer 0.05 and .10 to 1.0ul, in 0.10ul steps, of the concentrated dye solution into 3ml of distilled water brought to pH 9.9 with dilute NaOH. This represented concentrations of 0.667 to to 13.329 ug/ml (dilution factor included). Great care was taken to wipe solution from the needle without wicking. The optical density at 540nm was determined for the serial dilutions and the syringe transferred solutions. The theoretical and observed optical densities were compared to determine the actual volume of dye solution transferred. The 95% confidence limits for four independent replications were determined.

Determination of binding

Reaction vials of 5ml volume with caps and Teflon lined septa were purchased from Supelco (Supelco Park, PA). Small stirring bars were obtained from Fisher. The protein solutions were equilibrated at 20C before loading 1.00ml into the reaction tubes. For ligand concentrations ranging from 100 to 1000 ppm, the corresponding volume of ligand was carefully added to the reaction tube and the tube was tightly capped. The additional volume added was ignored in calculations. For concentrations from 1 to 50 ppm, a 100 ppm solution of hexanal in buffer was prepared by adding 10ul hexanal to buffer in a 100ml volumetric flask previously brought to volume and containing an added stir bar (Buttery et al., 1969). The volumetric flask was chosen to have a minimum headspace area. The solution was stirred overnight. The protein solution (0.5ml, 20 mg/ml) was loaded into a vial and a combination of buffer and ligand-containing buffer were

added to produce the desired concentration. The ligand solution was added last, and new solutions were prepared for each of three replications.

The equilibration time was determined for hexanal at concentrations of 5, 100 and 500 ppm. The headspace concentrations of the protein solutions and control, containing buffer but no protein, were determined after equilibration by using gas-liquid chromatography (GC). A 100ul gas tight syringe with replaceable plunger tip was obtained from Hamilton (Reno, NV). The syringe was kept at 35C between injections and was cleaned by using a heated vacuum syringe cleaner. The headspace was sampled by withdrawing 100ul of headspace gas and immediately injecting onto the GC. The vial was sampled 2 more times where 100ul of ligand-free gas was injected into the vial to prevent vacuum formation. The plunger tip was replaced at least once daily to ensure reliability. A Varian model 2400 gas chromatograph (Sunnyvale, CA) was equipped with a flame ionization detector and a 30m (0.72mm i.d.X 30m) bonded DB-1 Megabore column (J&W Inc., Rancho Cordova, CA) isothermal at 100C with nitrogen carrier gas. The gas flow rates were 15.5, 30, and 250 ml/min for nitrogen, hydrogen and air, respectively. Peak quantitation was done with a Beckman 427 plotting integrator. The detector and injector temperatures were held at 150C.

The protein molarity was calculated from the protein concentration by using molecular weights of 160 and 320 KDa, respectively.

Effect of reducing agent on binding

The binding with and without 10 mM 2-mercaptoethanol in the buffer was determined. This concentration is commonly used to store the soy proteins extracted in laboratory preparations. The 2-mercaptoethanol was added to buffer, protein, and hexanal solutions just prior to loading the vials.

Effect of ionic strength on binding

The effect of increased ionic strength was determined by adding 0.5M NaCl to the buffer. The levels of ligand below 100 ppm were prepared with the NaCl molarity increased sufficiently to take into account the dilution due to the addition of the ligand solution, which was in 30 mM Tris buffer.

Effect of sodium azide on binding

Sodium azide (0.02%) was added to the buffer and protein solutions. The azide was added to the protein, buffer, and hexanal solutions just prior to loading of the vials.

Determination of protein surface hydrophobicity

The method reported by Kamata et al. (1984) was used. A final volume of 4ml was prepared to contain 8mg protein, 1.25×10^{-4} M 1,8-anilinonapthalosulfonic acid (ANS), and 30mM Tris buffer, pH 8.0. The tubes were incubated for three hours. The fluorescent emission was recorded at 475 nm after excitation at 395 nm. The hydrophobicity of glycinin and B-conglycinin were determined after reaction with four ligands of differing functional groups, hexane, hexanal, hexanol, 2-hexanone, at four different levels: 0, 5, 100, 500, and 1000 ppm. The

experiment was treated as a 4X4 factorial. The hydrophobicity was determined for glycinin and B-conglycinin before and after heating the proteins at 100C for 4 minutes, to determine if surface hydrophobicity increased with protein unfolding. Experiments were done in duplicate.

Determination of reactive lysine

The dye binding method of Perl et al. (1985) was used with the following modification. A buffered protein solution (3ml of 50mg/ml) was used instead of dry protein. The reacted solutions were filtered through a 0.45uM filter before dilution and absorbance reading. Briefly, the method involves reaction of the protein with Orange G dye before and after reaction with proprionic anhydride (to remove nonspecific binding). The results were calculated as change in absorbance in samples reacted with ligand, 50 and 500ppm hexanal, and unreacted protein. A decrease in the absorbance of the dye solution indicates that less dye is bound to the protein; i.e., less reactive lysine.

Extraction of bound lipid

All solvents were reagent grade and were redistilled in glass before use. About 200 mg (5ml) of the concentrated isolated soy fractions was extracted with chloroform-methanol (50ml, 2:1, v,v) by using a blender. The emulsions which formed were allowed to separate overnight in separatory funnels. The organic phases were filtered through Celite and the solvent was removed under vacuum. The lipid obtained was dissolved in 20ul chloroform.

Determination of lipid classes

The lipid classes triglyceride, free fatty acid and polar lipid were determined by using the thin layer chromatography flame ionization Iatroscan system. The rods were spotted with lul of the extracted lipid. Standard phosphatidyl choline and stearate were obtained from Sigma (St. Louis, MO). Corn oil was used as a triglyceride standard. About 10ug of each standard was spotted per rod. The solvent system was hexane/diethyl ether/acetic acid (97:3:1 v,v). The polar fraction was obtained by developing a standard TLC plate, spotted with the lipid, with 80:20:1 (hexane/ethyl ether/acetic acid) and extracting the silica gel, scraped from the origin, with chloroform. The phospholipid class were separated in the polar fraction by using the method of Innis and Clandinin (1981). The solvent system used was chloroform/methanol/water (80:35:4, v,v). Standard phosphatidyl ethanolamine and phosphatidyl inositol were obtained from Sigma (St. Louis, MO).

Results and Discussion

Syringe calibration

The syringe calibration indicated that the 95% confidence limit for the actual amount of dye transferred encompassed the theoretical value from 0.05 to 0.60ul. Above 0.60ul, the theoretical amount was lower than the 95% confidence limit of the actual amount. The percentage of the theoretical value which was actually transferred above 0.60ul ranged from 102 to 104%. These correction factors were

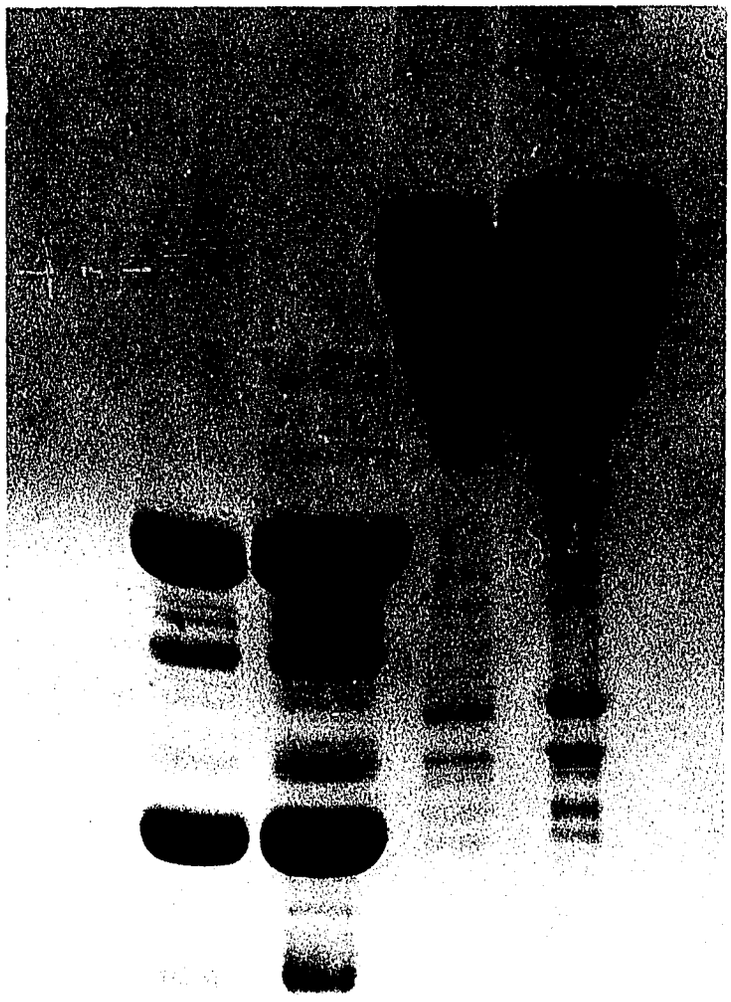
used in the data analysis. The coefficient of variation (COV) of four replications was below 1.3 % for all volumes transferred except 0.05ul, where it was greater than 5%; therefore, this level of transfer was not used. The regression of COV against level of dye transferred from 0.10 to 1.0ul was not significantly different from zero.

Protein purity

The SDS-PAGE showed that the proteins preparations were very pure. Only very minor contaminating bands were observed even at high protein loads (Figure 1). The modification of the Thanh and Shibasaki isoelectric precipitation procedure increased the purity of the crude B-conglycinin fraction, but the yield was much lower. The immunoelectrophoresis indicated that there was no immunoreactive glycinin in the B-conglycinin preparation and the glycinin preparation contained only 0.06% B-conglycinin. The protein load used to determine extremely small amounts of cross-contamination was 100ug. Under the conditions employed, the minimum detectable amount of protein was about 0.025ug.

The duration of the dialysis procedure was 5 days to ensure that the buffer transfer was complete and sodium azide and 2-mercaptoethanol were removed. The 24 hour dialysis of diluted protein was done to ensure that the concentrated protein did not coat the inner surface of the dialysis bag and inhibit equilibrium. Enough protein was processed at one time for two weeks of experiments. A total plate count and psychrophile count at the end of two weeks of

Figure 1. Polyacrylamide electrophoresis of purified soybean glycinin (left lanes) and B-conglycinin (right lanes)



refrigerated storage indicated that bacterial growth was not a problem in the proteins (both counts were below 500 organisms/ml).

Lipid bound to purified proteins

The Iatroscan analysis of the extracted lipid indicated that polar lipid predominated, although trace amounts of triglycerides and free fatty acid also were present. The estimated quantity of polar lipid was 2.23 and 1.49 mg/g for glycinin and B-conglycinin respectively. The Rf values for PC, PI and PE, separated by using the method of Innis and Clandinin (1981), were 0.11-0.19, 0.41 and 0.65-0.68, respectively. The differences in Rf values obtained for a particular compound reflect differences in spotting height and total solvent migration within a group of rods. The polar lipid from the protein samples was separated into one major peak with Rf 0.68-0.70 which corresponded to over 90% of the total area and one minor peak at Rf 0.08, for both glycinin and B-conglycinin. The major peak is undoubtedly not PC, which is strongly retained by the rods. The minor peak may be PC. The possibility that the polar lipids are glycolipid can not be discounted. The minor amount of polar lipid material bound to the protein would likely only be removed completely by using solvents more polar than the alkanes commonly employed. The effect of bound lipid on ligand binding would be difficult to establish because removal with polar solvents would likely involve protein denaturation. An unambiguous identification of what polar lipid class(es) are involved would allow the study of the effects of addition of small amounts of lipid to the protein, and the effects of this binding on

ligand protein interactions.

Binding of hexanal to glycinin and B-conglycinin

The linear regression of the repeated sampling of the headspace vials revealed no significant change in headspace concentration over 5 injections at 50, 500 and 1000ppm hexanal (F=0.43, 1.06, 0.31, respectively). This indicated that the amount of dilution which took place did not effect the headspace concentration.

The binding curve obtained at 30°C is illustrated in Figure 2a. Saturation of the binding sites seems to have been obtained. The double reciprocal plot of these data is shown in Figure 2b. The curvature of the double reciprocal plot indicates that positive cooperativity is present. The modified double reciprocal plot can indicate the minimum number of classes of sites involved in the cooperative effect (Thissen, 1982; Segal, 1975). In this modification, the reciprocal of the free ligand concentration is brought to different powers; the power that produces a straight line is the apparent (minimum) number of cooperating classes of sites. Segal (1975) pointed out that this number is not necessarily an integer and is a minimum number because a strong cooperativity is assumed. The correlation coefficients, obtained for different powers, indicated that the apparent number was about 1.3 for B-conglycinin and 1.5 for glycinin. The fact that the numbers are not integers probably indicates that there are two classes but the cooperativity is not high.

The number of binding sites per protein molecule can be estimated

Figure 2. Binding of hexanal to glycinin and B-conglycinin

A. Saturation curve

B. Double reciprocal curve

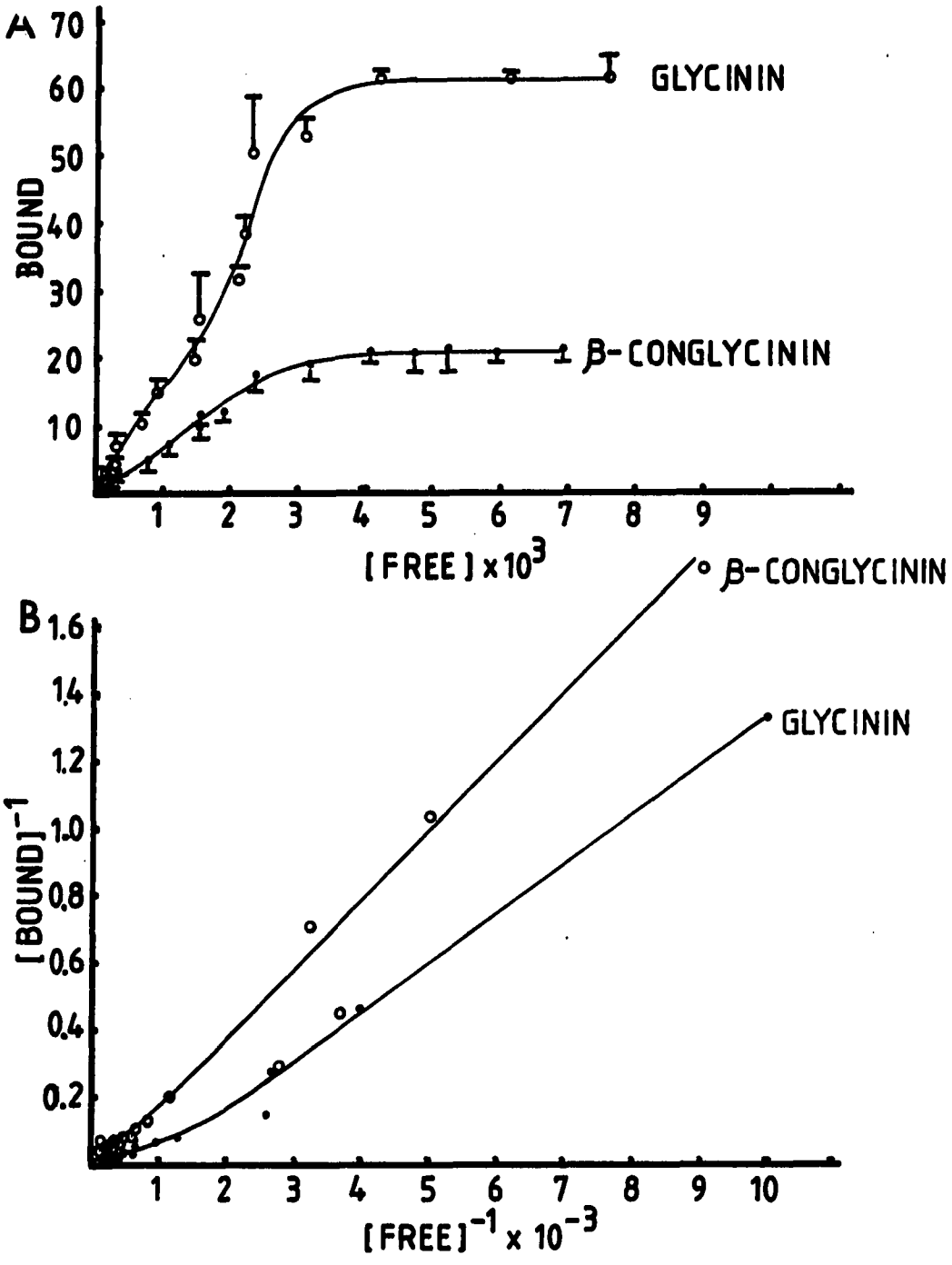

A
BOUND
70
60
50
40
30
20
10
GLYCININ
β-CONGLYCININ
1
2
3
4
5
6
7
8
9
[FREE] x 10^3
B
[BOUND]^-1
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
β-CONGLYCININ
GLYCININ
1
2
3
4
5
6
7
8
9
10
[FREE]^-1 x 10^-3

Table 1. Binding parameters for hexanal to glycinin and B-conglycinin reacted under different conditions

Protein condition	graph fit n	graph fit K	Klotz plot n	Klotz plot K	Wilkinson (1961) n	Wilkinson (1961) K
Glycinin						
Tris .03M	60	555	84	483	108±10.2	270 ±24.1
0.5M NaCl	33	200	43	172	38±2.9	238 ±32.3
0.02% NaN_3	77	1075	77	1172	76±2.6	1250 ±182.6
10 mM BME	62	500	122	180	112±10.3	213 ±24.1
B-conglycinin						
Tris .03M	21	175	23	1437	35±2.6	303 ±30.3
0.5M NaCl	22	500	26	1431	68±7.6	125 ±13.9
0.02% NaN_3	29	500	40	255	32±2.4	417 ±83.3.
10 mM BME	20	625	36	235	32±2.0	303 ±32.7

from the saturation plot to be about 60 and 21 for glycinin and B-conglycinin, respectively. The double reciprocal plot binding constant and number of sites were quite different as determined by linear regression using double reciprocal and the method of Wilkinson (1961) and are presented in Table 1. The number of binding sites obtained from the Wilkinson procedure are higher than estimated from

the saturation plots; 107.5 ± 10.2 and 34.6 ± 2.6 for glycinin and B-conglycinin, respectively. Because of the curvature of the double reciprocal plot, the parameters could not be estimated from the regression due to the negative Y-intercept obtained. The affinity constants obtained indicate that the affinity for hexanal was slightly greater for B-conglycinin than glycinin; respectively, 303 ± 30.3 and 270 ± 24.1. A Student T-test using a pooled variance indicated that there was no significant difference between the affinity constants ($P<0.05$, $df=(n-2)=4$).

The binding parameters were calculated for B-conglycinin using only the 7 lowest of the 17 data points, to determine if using a partial binding curve greatly affected the results obtained. The number of binding sites obtained was 24 ± 6.6, and binding constant was 400 ± 77.4. Thus, using a partial curve greatly affected the parameters which were determined; the number of sites decreased whereas the binding constant increased.

The turbidity of the soy proteins which were reacted with hexanal increased at high levels of hexanal (>300ppm). The turbidity of the glycinin solutions was much higher than B-conglycinin. Initial immunoelectrophoretic analysis of the proteins reacted with different levels of hexanal indicated that the immunoreactive protein decreased with increasing level of ligand; however, a more careful study indicated that this finding was a result of precipitation of the protein which became insoluble with high levels of ligand concentration. The immunoreactive protein did not change in solutions

which were carefully mixed prior to dilution and immunoelectrophoresis; the slope of the regression of immunoreactive protein with ligand concentration was not significantly different from 0. This finding that turbidity and immunoreactivity were not related is supported by Guzman and Murphy (1988).

ANS surface hydrophobicity

The surface hydrophobicity was determined before and after heating the purified protein solutions on a boiling water bath for 4 minutes. The increase in hydrophobicity was much greater for glycinin than B-conglycinin; the ratios of fluorescent intensity after/before heat treatment were 4.61±0.101 and 2.06±0.095, respectively. This agrees with Guzman and Murphy (1988) who found that B-conglycinin structure was much more heat stable than glycinin in a highly purified system devoid of reducing agent by using an immunoelectrophoretic method. The increase in turbidity after heating was much greater for glycinin than B-conglycinin.

The ligand functional group has been previously been shown to be important in the binding to soy proteins in aqueous systems (Thissen, 1982; Gremli, 1974; Beyeler and Solms, 1974; Arai et al., 1971). The surface hydrophobicity of glycinin and B-conglycinin, reacted with 5 levels of ligands with different functional groups, is presented in Table 2. The ANOVA results are shown in Table 3. The ANOVA for B-conglycinin indicates that there were no significant differences from any of the sources whereas glycinin showed significant ligand, linear, and interaction effects. Most of the level sums of squares were found

in the linear source, as was the case with the interaction. Because of the significant interaction, linear regressions were performed for each ligand separately with level, and the results are presented in Table 4. In spite of the nonsignificant level effect for B-conglycinin, the regression of hexanal with level was significant ($P<0.01$, df=5) for both glycinin and B-conglycinin. The linear effect of the ANOVA is a regression of all four ligands with level and was not significant for B-conglycinin, apparently because three of the four ligands did not fit a linear model. Level for B-conglycinin approached significance ($P<0.0805$) but the interaction was outside the 10% significance level ($P<0.1113$). The ligand functional group, thus, seems to be important in the ligand mediated increase in surface hydrophobicity. The reason for this is unknown.

Orange G reactive lysine

The decreases in absorbance, indicating loss of reactive lysine, after reaction with hexanal at 50 and 500ppm were very minor; glycinin 0.018 ± 0.006, 0.015 ± 0.003, B-conglycinin $0.038\pm.009$, 0.025 ± 0.009, respectively. There were no significant differences between ligand level or between proteins as determined by a T test with pooled variance. The changes in absorbance were greater for glycinin than B-conglycinin but the changes were small, ranging from 0.013 to 0.045. A T test indicated that the observed absorbances were significantly different from 0 at the 5% level (calculated T=6.25, df=7). Perl et al. (1985) reported the sensitivity of this method was sufficient to detect a 5% change in reactive lysine.

Table 2. Surface hydrophobicity of glycinin and B-conglycinin after reaction with varying levels of ligands with differing functional groups

Protein	ppm	ligand			
		hexanal	hexanol	2-hexanone	hexane
B-conglycinin	0	162.6[a]	162.7	163.6	162.7
		0.57[b]	0.85	0.14	0.92
	5	162.2	164.0	163.1	160.8
		1.27	0.14	5.37	0.57
	100	160.8	161.2	159.6	162.5
		0.85	1.20	6.43	1.06
	500	165.6	161.3	164.4	159.3
		1.77	2.05	3.25	2.83
	1000	168.3	163.9	162.3	162.6
		2.05	0.35	3.39	3.68
Glycinin	0	86.3	87.1	86.8	87.3
		0.78	1.48	0.92	1.20
	5	85.5	85.6	85.9	85.9
		0.21	0.49	0.35	0.35
	100	88.5	84.3	84.2	86.7
		0.49	0.64	0.92	0.64
	500	91.9	83.8	87.8	84.4
		2.33	1.56	1.20	1.91
	1000	98.5	87.5	88.2	87.4
		0.14	1.91	0.21	1.34

[a] $\bar{X}$.
[b] s.d.

Table 3. Analysis of variance results of the ANS hydrophobicity of the different levels of the various ligands

Protein	source	df	SS	F	P>F
B-conglycinin	replication	1	0.60	0.09	NS
	ligand	3	27.07	1.30	NS
	level	4	43.49	1.56	NS
	linear	1	21.69	3.12	NS
	quadratic	1	7.19	1.03	NS
	lack of fit	2	14.62	1.05	NS
	ligand*level	12	88.60	1.06	NS
	ligand*linear	3	43.38	2.08	NS
	ligand*quad	3	14.31	0.69	NS
	lig*LOF[a]	6	45.22	1.08	NS
	error	19	132.07		
Glycinin	replication	1	1.89	1.50	NS
	ligand	3	121.03	32.05	.01
	level	4	113.53	27.55	.01
	linear	1	95.61	75.97	.01
	quadratic	1	12.26	9.74	.01
	lack of fit	1	5.66	4.50	.05
	ligand*level	12	166.20	11.00	.01
	ligand*lin	3	138.33	32.05	.01
	ligand*quad	3	13.06	3.46	.05
	ligand*LOF	6	14.81	1.96	NS
	error	19	23.92		

[a]LOF: lack of fit.

Table 4. Regression coefficients of surface hydrophobicity of individual ligands with level (error df=5)

Ligand	Protein	
	glycinin	B-conglycinin
hexanal	0.978[a]	0.887[a]
hexanol	0.229	0.172
2-hexanone	0.667	0.047
hexane	0.342	-0.001

[a] $p<0.01$.

The effect of 0.5M NaCl

The binding of hexanal to the proteins in the presence of 0.5M NaCl is illustrated in Figures 3a and 3b. The salt increased the headspace concentration in the buffer as well as the protein tubes. The results from the Wilkinson procedure are shown in Table 1 and indicate that the presence of NaCl decreased the affinity of hexanal for B-conglycinin but had no significant effect for glycinin. The number of binding sites decreased to 37.5 ± 2.85 for glycinin but increased for B-conglycinin to 68.4 ± 7.60. The saturation plot (Figure 3a) seems to indicate that the number of binding sites is

similar for the two soy proteins, as saturation seems to have been obtained around 22-23 moles bound per mole protein. The double reciprocal plot (Figure 3b) illustrates that the curvature in the glycinin and B-conglycinin plots is enhanced in the presence of NaCl. Damodaran and Kinsella (1981a) reported that, in a model system containing 0.5 M NaCl, glycinin had 8 binding sites with a binding constant 290 M^{-1} on the basis of 100 KDa molecular weight. The number of sites would be about 26 if the correct molecular weight were used. The modified Klotz procedure indicated that a minimum of 1.5 classes were present for both proteins. Again, this is possibly due to two classes having weak cooperativity.

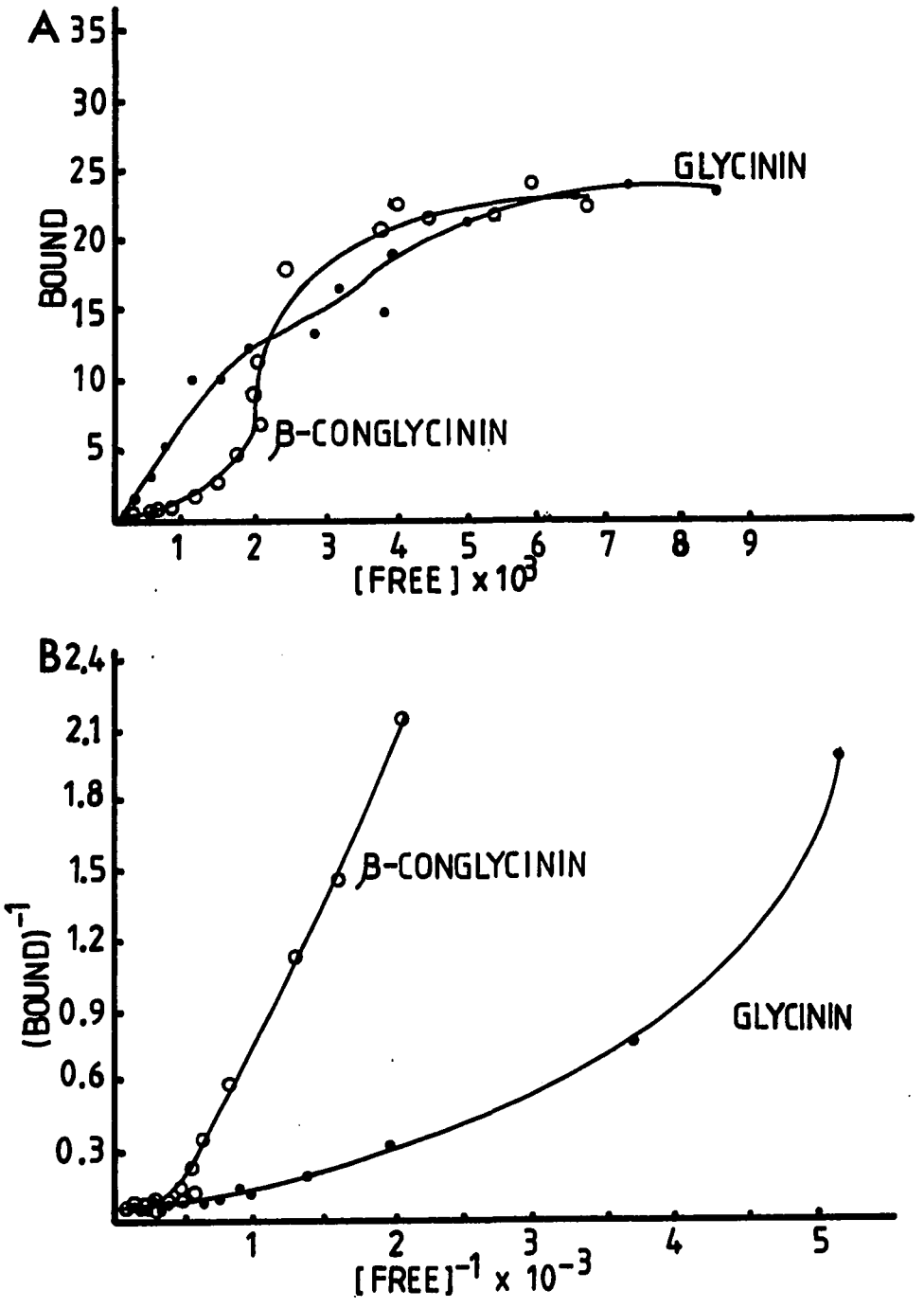
Effect of sodium azide

The binding curves in the presence of 0.02% NaN_3 are illustrated in Figures 4a and 4b. Comparison of the saturation curves with those obtained in buffer alone illustrates that the binding for B-conglycinin differed very little, whereas for glycinin the binding increased more rapidly and appeared to reach saturation at a higher level in the presence of sodium azide. The binding constants obtained, 1250 and 417 for glycinin and B-conglycinin, respectively, are higher than buffer alone while the number of binding sites decreased for glycinin but not B-conglycinin. The double reciprocal plots did not display the curvature observed in the absence of sodium azide. This could be due to the fact that data for binding below 50ppm were not obtained for the model system containing sodium azide.

Figure 3. Binding of hexanal to glycinin and B-conglycinin in model system containing 0.5M NaCl

A. Saturation curve

B. Double reciprocal plot


A
35
30
25
20
15
10
5
BOUND
GLYCININ
β-CONGLYCININ
1
2
3
4
5
6
7
8
9
[FREE] x 10³
B
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
(BOUND)⁻¹
β-CONGLYCININ
GLYCININ
1
2
3
4
5
[FREE]⁻¹ x 10⁻³

In the model systems where positive cooperativity was observed, the curvature was due to the differences in binding at low ligand concentrations; if the data from 150-1000 ppm were examined, there would be no curvature in the double reciprocal plots. In studies where only a small portion of the binding curve is examined, the curvature in the double reciprocal plot may be missed. The reason for the increased binding affinity for glycinin in the presence of sodium azide is unknown. The results are surprising because Damodaran and Kinsella (1981a) reported a low affinity of 2-nonanone to glycinin in the presence of sodium azide.

Effect of 2-mercaptoethanol

The binding of hexanal to soy proteins in the presence of 2-mercaptoethanol is illustrated in Figure 5 and the binding parameters presented in Table 1. The number of binding sites and binding constants were not significantly different for either protein, compared to the model system without mercaptoethanol. The mildly reducing conditions apparently had no effect on the binding.

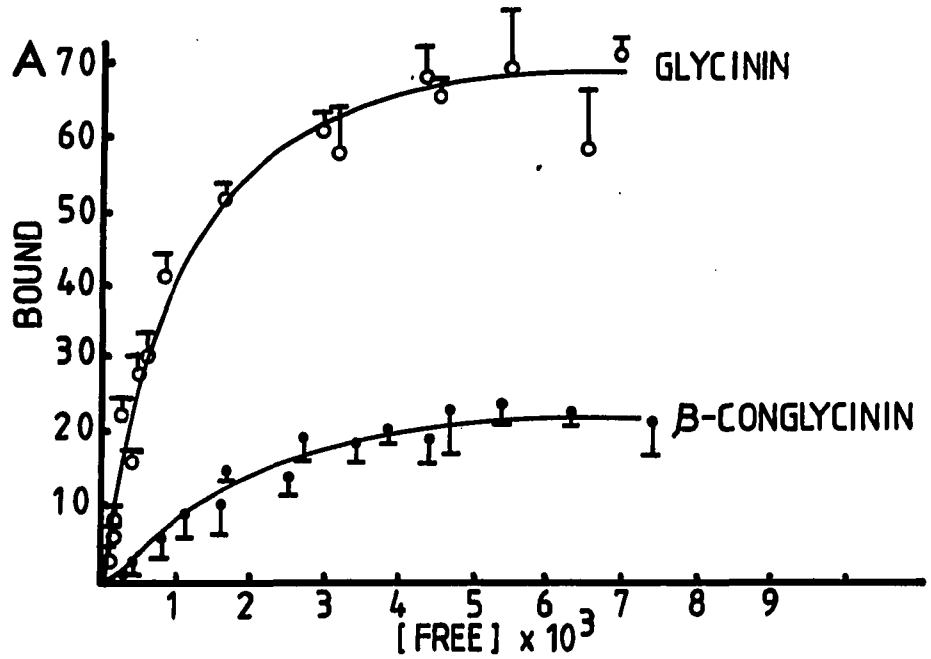
Conclusions

The binding of hexanal to glycinin and B-conglycinin as determined in this study differs from results presented by Damodaran and Kinsella (1981b) and O'Neill and Kinsella (1987). The numbers of binding sites determined, 107.5 and 34.6 for glycinin and B-conglycinin, were much higher than reported by Kinsella and coworkers but is closer to the 81 sites reported by Thissen (1982), and the

Figure 4. Binding of hexanal to glycinin and B-conglycinin in a model system containing 0.02% sodium azide

A. Binding curve

B. Double reciprocal plot


A
BOUND
70
60
50
40
30
20
10
GLYCININ
β-CONGLYCININ
1
2
3
4
5
6
7
8
9
[FREE] x 10³

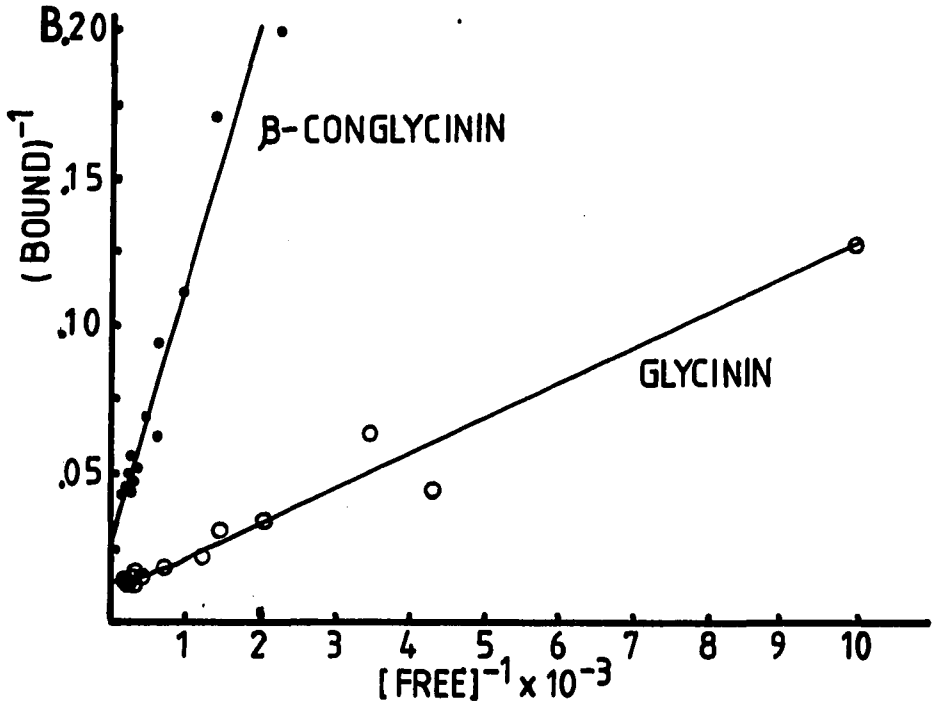
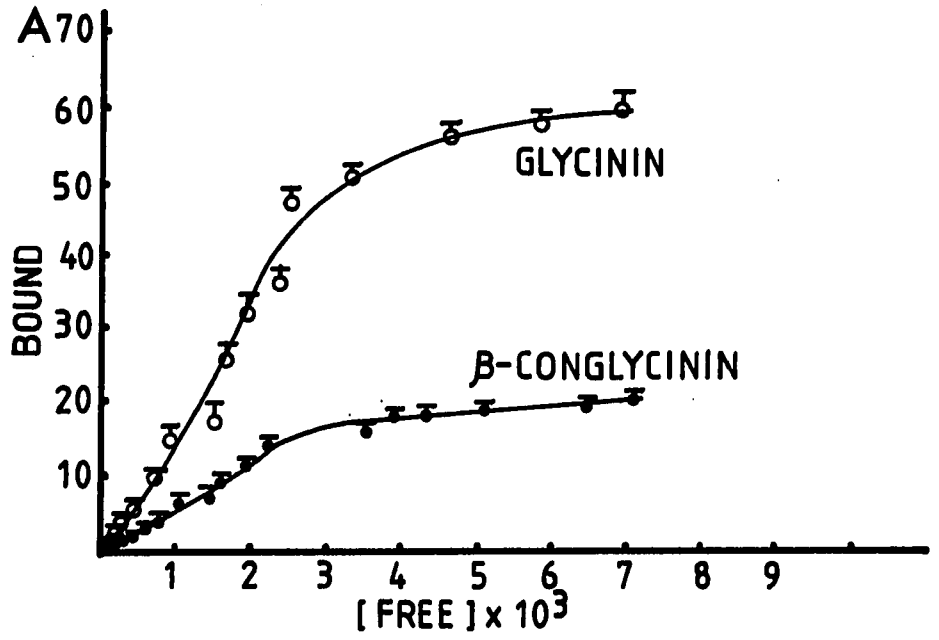
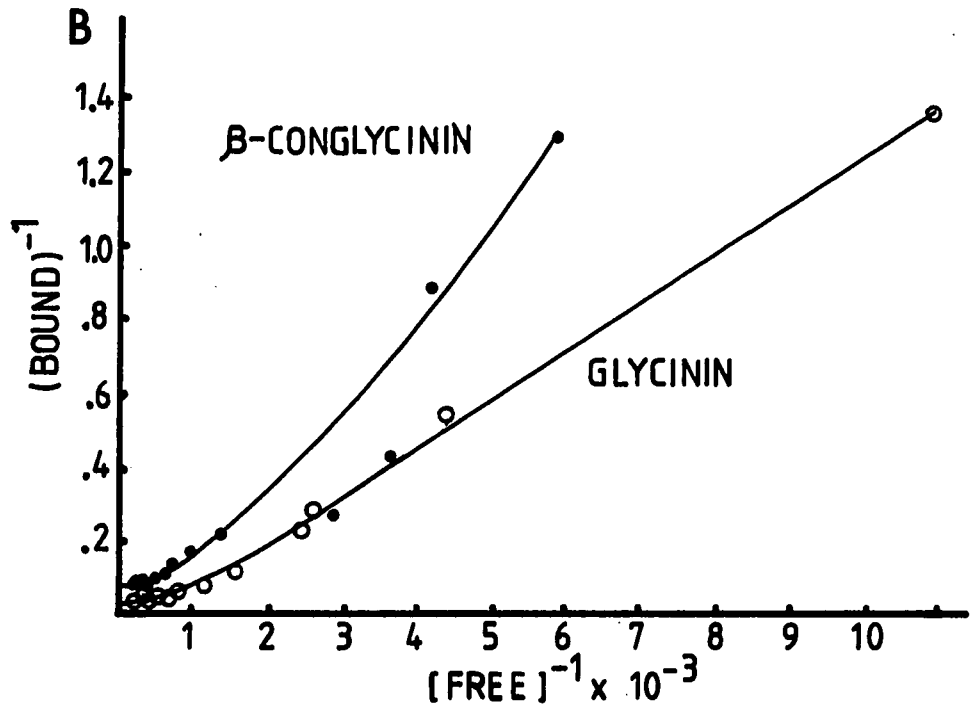

B
(BOUND)⁻¹
.20
.15
.10
.05
β-CONGLYCININ
GLYCININ
1
2
3
4
5
6
7
8
9
10
[FREE]⁻¹ x 10⁻³

Figure 5. Binding of hexanal to glycinin and B-conglycinin in a model system containing 10mM 2-mercaptoethanol

A. Binding curve.

B. Double reciprocal plot.


A
BOUND
70
60
50
40
30
20
10
GLYCININ
β-CONGLYCININ
1
2
3
4
5
6
7
8
9
[FREE] x 10³


B
(BOUND)⁻¹
1.4
1.2
1.0
.8
.6
.4
.2
β-CONGLYCININ
GLYCININ
1
2
3
4
5
6
7
8
9
10
[FREE]⁻¹ x 10⁻³

estimated minimum of 27 binding sites for heptanal binding to soy isolate calculated from the data presented by Gremli (O'Keefe, 1988). The data of Arai et al. (1970) indicate, however, that fewer than two binding sites were present in the soy protein which was studied. The differences in the data may be due a number of factors: the soy proteins came from different sources, have undergone different treatments, and were of different purities; the presence of sodium azide which has been shown to effect the binding parameters; the use of partial binding curves to obtain data; and finally, the different techniques which have been employed to determine binding parameters may have some role in the reported differences.

The reason for the effect of NaCl on binding could be due to the known effect NaCl has on the tertiary structure of the multimeric soy proteins, as suggested by Damodaran and Kinsella (1981a), and/or the effect of the salt on ligand binding. Salt would be expected to increase the strength of hydrophobic bonds but a decrease in binding constant was observed for B-conglycinin. It is perhaps necessary to investigate smaller changes in ionic strength, which would not have as great an effect on the protein tertiary structure, to determine if increasing salt concentration affects the hexanal binding in the absence of protein structural changes.

The ANS hydrophobicity and turbidity measurements seem to indicate that structural changes occur as a result of hexanal binding. The positive cooperativity observed for binding indicates that the structural changes may affect the binding process. The small

decreases in Orange G reactive lysine are not striking, however, they illustrate that there may be covalent binding occurring between the aldehyde group and the lysine amino, but a more sensitive test is necessary. The lack of significant changes in the immunoreactivity of protein solutions which were obviously turbid indicates that the structural changes which caused greater light scattering did not cause the epitopes for antibody recognition to become unavailable. This could be due to the degree of denaturation which may occur because of hexanal binding.

It would be an oversimplification to suggest that we now have a good understanding of carbonyl protein interactions. The binding is assumed to be reversible for our data calculations. This has not been proven. Because we have found turbidity changes and insolubilization of the protein, a true reversible equilibrium may not have been reached. If this is the case, a different technique than equilibrium dialysis would be necessary.

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PART II..

EFFECT OF TEMPERATURE ON THE BINDING OF A SERIES OF VOLATILE FLAVOR COMPOUNDS TO SOY PROTEINS IN AN AQUEOUS MODEL SYSTEM

Introduction

The binding of carbonyl flavor compounds to soy proteins can cause unacceptable beany flavors in products containing the soy proteins. The binding of desirable flavors, for example chicken flavor in a formulated soup (Malcolmson et al. 1987), or meat flavors in texturized meat substitutes (Van den Ouweland and Schutte, 1978), can cause problems in determining the appropriate level of flavoring. Interactions of soy proteins with spice flavor compounds may alter the flavor profile of the product and lead to new off-flavors (Schutte and Van den Ouweland, 1979).

> "Knowledge of the magnitude of the energy, entropy, and heat capacity changes involved in ligand binding equilibria of biological compounds is essential for a prediction of the thermodynamic properties of the system under different environmental conditions" (Wiesinger and Hinz, 1986).

In other words, knowledge of the forces involved in flavor protein interactions may allow the formulation of flavorings which do not interact with the soy proteins and may facilitate removal of the

bound compounds causing beany flavors.

The basic thermodynamic parameters are the Gibbs free energy (ΔG), entropy (ΔS), and enthalpy (ΔS). The symbol Δ indicates a change in the observed parameter. The ΔG will indicate whether or not the reaction will occur spontaneously; a negative ΔG indicates that the products of the reaction, the ligand bound protein, will be favored. At equilibrium, the ΔG is equal to zero. The ΔG^{o} is the difference in free energy between products and reactants when they are in their standard states.

A number of reports have investigated the thermodynamics of flavor protein interactions. The interactions of a series of aldehydes, ketones, methyl esters, alcohols, and hydrocarbons with soy protein isolate was examined by Aspelund and Wilson (1983). The heats of adsorption were determined for dry soy protein isolate by the retention of the compounds injected onto gas liquid chromatography (GLC) columns packed with the soy protein. The authors used statistical analysis to determine if linear regression (of ln retention time with the reciprocal of the absolute temperature) fit the data, slope and error estimates, and if the slopes determined for the regression were significantly different from zero. The data indicated that the enthalpy of the reaction was the driving force. The functional group was important in the binding and the authors used the thermodynamic data to postulate the types of bonds which occurred for the different compounds; hydrocarbons bound with van der Waals force only, whereas, in addition, alcohols formed two hydrogen bonds

and the ketones, aldehydes, and methyl esters formed one hydrogen bond.

Thissen (1982) determined the thermodynamic parameters for the interaction of carbonyls with an aqueous solution of soy protein. The method employed was eqilibrium headspace concentration. The bound ligand was determined by comparison of headspace ligand concentrations between a blank and soy protein solution. The driving force for the interactions was determined to be the entropy. This indicates that the increase in the disorder of the system, greater after binding, was more important than the heat change. A positive cooperativity in binding was reported. This indicates that the binding of flavor compounds increased the binding for further compounds. This was interpreted as an unfolding of the protein, exposing new binding sites. Hexane was found to have no affinity for the soy protein. This is surprising, if in fact the binding is simply hydrophobic. But the functional group has been previously reported to be important in the binding of flavor compounds to soy proteins (Solms et al., 1973; Beyeler and Solms, 1974; Gremli, 1974; Arai et al., 1970).

Damodaran and Kinsella (1981a) investigated the interactions of 2-heptanone, 2-octanone, 2-nonanone, 5-nonanone, and nonanal to soy protein isolate. The Gibbs free energy (ΔG^{o}) increased, became more negative, as the chain length of the 2-ketones increased. This was interpreted as suggesting that the binding was simply hydrophobic. The authors concluded that the lower binding energy of 5-nonanone, compared to 2-nonanone and nonanal, was due to the interference the

carbonyl group had on the hydrophobic binding. Some of the problems with the equilibrium dialysis approach, used by Damodaran and Kinsella, were discussed by Wilson (1985, 1986). The binding is assumed to be reversible, as isooctane extraction of the protein solution was used to separate the protein from ligand. This aspect of binding has not been carefully studied, although there are reports that some of the binding is irreversible (Gremli, 1974). The formation of emulsions, in the organic solvent extraction of solutions containing protein, causes problems of phase separation and inclusion of ligand in the solvent-denatured protein. It cannot be concluded, as a result of a partition coefficient determined for buffer and organic solvent, that the same extraction efficiency will be obtained in the presence of a protein. King and Solms (1979) reported that benzyl alcohol was reversibly bound to soy protein, but it is unlikely that the more reactive aldehydes and ketones will behave similarly; the facile formation of amino-carbonyl bonds between hexanal and protein has been reported (Okitani et al., 1986).

Hydrophobic bonding is usually considered to be driven by an increase in the randomness of the system because of the reduced amount of highly structured water which surrounds nonpolar compounds in aqueous solution; however, Connors (1987) points out that hydrophobic bonding may be entropy or enthalpy driven. It is also possible that an unfolding of the protein structure, because of ligand binding, would contribute to a change in entropy. The interior of protein is usually characterized by a high concentration of nonpolar groups

which, due to their hydrophobic bonding, contribute to protein stability. The forces involved in protein ligand binding are of the same order of those stabilizing the protein structures (Wiesinger and Hinz, 1986). It is, therefore, not surprising that conformational changes in proteins after ligand binding have been reported (Damodaran and Kinsella, 1980) or suggested (Solms et al., 1973; Thissen, 1982).

The binding of meat flavor compounds to soy glycinin and B-conglycinin was studied by Einig (1983). The free energy changes were negative, indicating that the reactions are thermodynamically favorable. The increase in entropy was reported to drive the reaction. The binding was reported to be via hydrophobic and electrostatic bonding. Methional was found to be completely retained by the soy proteins under the conditions studied.

The two main storage proteins in soybeans are glycinin and B-conglycinin (Nielsen, 1985). There is evidence that B-conglycinin has a much greater affinity for 2-nonanone than glycinin, although the number of binding sites is greater for glycinin (Damodaran and Kinsella, 1981b; O'Neill and Kinsella, 1987). The method used by Damodaran and Kinsella (1981b) to purify glycinin and B-conglycinin has recently been reported to provide purities of about 50 to 70 %, respectively (Iwabuchi and Yamauchi, 1987). 2-nonanone is a rather unfortunate choice as a model carbonyl compound because of it is not one of the flavor compounds involved in the beany flavor problem (Rackis et al., 1979; Cowan et al., 1973), is present in very low concentrations in soy products (del Rosario et al., 1984), and

differences between the binding of ketones and aldehydes to soy protein have been reported (Gremli, 1974; Beyeler and Solms, 1974). The presence of sodium azide and 2-mercaptoethanol in the buffer used by Kinsella and coworkers leads to questions of the applicability of the determined binding parameters to food systems (Wilson, 1986) because of the effect of these compounds on binding (O'Keefe et al., 1989); reducing agents often alter protein-ligand equilibria (Wiesinger and Hinz, 1986). A study using taste panel evaluation of soy-extended beef patties indicated that meat flavor intensity was reduced by isolated soy protein as well as by soy glycinin or B-conglycinin, although the nonmeat aroma was not increased (Einig, 1983). There were no differences between glycinin and B-conglycinin. Thus, the suggestion that soy glycinin would not bind flavor compounds and therefore would have greater application in food products (Damodaran and Kinsella, 1981b) seems doubtful.

The purpose of this study was to use a headspace technique to determine the thermodynamic parameters for the binding of a series of aldehydes, ketones, alcohols, and hexane to purified soybean glycinin and B-conglycinin.

Materials and Methods

Compounds studied

The carbonyl compounds examined included aldehydes (butanal, pentanal, hexanal, heptanal, octanal), ketones (2-hexanone, 3-hexanone, 2-nonanone, 5-nonanone), 1-hexanol and hexane. The

compounds were obtained from Aldrich or NuCheck. The aldehydes and ketones were stored at -20°C. The purities were checked by gas chromatography and were greater than 97%.

Gas liquid chromatography

The column used was a DB-1 bonded Megabore (30M X 0.53mm i.d.) obtained from J&W (Rancho Cordova, CA) operated in a Varian model 2400 (Sunnyvale, CA) gas chromatograph (GLC) equipped with a flame ionization detector. The flow rates for hydrogen, air, and nitrogen carrier gas were 30, 250, and 15.5 ml/min, respectively. The column was operated in a GLC equipped for packed columns with no injection split or detector make-up gas. The high carrier gas flow rate with nitrogen was obviously not chosen to maximise efficiency, however the number of theoretical plates was typically around 10,000. The void volume was 9.0 ml and linear flow velocity was 0.86 m/sec, calculated by using methane gas. Column temperature ranged from 50 to 150C. The higher molecular weight carbonyls required an elevated column temperature to obtain reasonable retention times (below 5 minutes). The capacity ratio (k') ranged from 0.9 to 5 for the compounds studied. The syringe was cleaned between injections with a heated vacuum cleaning apparatus obtained from Hamilton (Reno, NV).

Determination of binding and thermodynamic parameters

The ΔG° was calculated from the equation: $\Delta G^{\circ} = -RT\ln K$. The ΔH° was determined from the Van't Hoff equation:

$\ln(K_2/K_1) = (\Delta H^{\circ}/R)(1/T_2 - 1/T_1)$, where the K is the binding constant, T is the absolute temperature in °Kelvin (°K), and R is the gas

constant. The ΔS^{o} was obtained after knowledge of ΔG^{o} and ΔH^{o} at a particular temperature by using the equation: $\Delta G^{o} = \Delta H^{o} - T\Delta S^{o}$.

The method of determination of binding constant and site numbers was described by O'Keefe et al. (1989).

Protein hydrophobicity

The method described by Kamata et al. (1984) was used to estimate the hydrophobicity of glycinin and B-conglycinin. A 2x2 factorial was designed with temperature (5^{o} and 20^{o}C) and level (0 and 100 ppm hexanal) effects. The results were examined by using the SAS system (Cary, NC).

Results and Discussion

The headspace technique allowed sampling of all the compounds studied. The only compounds which adsorbed onto the silanized syringe barrel were the nonanone isomers and, to a lesser extent, octanal. Nonanone retention by syringes has been previously reported by Buttery et al. (1969), who indicated that this adsorption would not preclude quantitative headspace sampling. The syringe cleaning procedure ensured that carryover was minimized. Examination of the soy proteins with no added ligand indicated that endogenous volatile compounds were present at extremely low concentrations. Identification of the compounds would require trapping or a special headspace sampling apparatus to increase the amount of the compounds injected onto the column. The usable range of ligand concentrations was limited at 5^{o}

compared to 20° and 30°C. At 5°C, the ligand levels below 40 ppm produced GC responses, at maximum sensitivity, which approached the baseline noise and endogenous volatiles which made quantitation impossible. The nonanone isomers, especially 5-nonanone, reached saturation at 5°C at levels which were usable at the higher temperatures. These factors limited the range of data which could be determined at 5°C. The determination of the binding parameters was attempted at 40° and 50°C; however, at these temperatures, condensation of water on the sides and septa of the vials was apparent. The presence of condensed liquid would cause problems in interpretation because of the micro-equilibria between the "distilled" water phase on the surfaces of the vials and the headspace.

The ANS surface hydrophobicity of B-conglycinin and glycinin at two temperatures and levels of ligand is shown in Table 1 and the ANOVA result in Table 2. It can be seen that the temperature was the only significant effect for both proteins, although level almost reached the 10% level. The regression of level with hydrophobicity for hexanal has previously been shown to be significant for both proteins (O'Keefe et al., 1989). The increase in hydrophobicity at 5° compared to 20°C has been previously hypothesized by Damodaran and Kinsella (1981) to explain a greater binding affinity of 2-nonanone at the lower temperature. It is possible that the lower temperature weakens hydrophobic bonds and allows protein unfolding.

Table 1. Surface hydrophobicity of glycinin and B-conglycinin determined with ANS. Effect of temperature and hexanal level on apparent surface hydrophobicity

Protein	hexanal level (ppm)	temperature	
		5	20
Glycinin	0	112[a] (2.97[b])	85.9 (3.25)
	100	120.5 (4.53)	88.9 (3.96)
B-conglycinin	0	243.7 (5.23)	162.4 (3.25)
	100	223.6 (14.00)	158.7 (4.67)

[a] mean.
[b] standard deviation.

Table 2. Analysis of variance of the 2X2 factorial of surface hydrophobicity with temperature and level effects

Protein	Source	df	SS	F	PR>F
B-Con	temperature	1	1687.8	121.5	0.0004
	level	1	61.6	4.4	0.1030
	temp*level	1	13.0	0.94	0.3888
	error	4	55.6		
Glycinin	temperature	1	10687.2	167.1	0.0002
	level	1	283.2	4.4	0.1031
	temp*level	1	134.5	2.1	0.2206
	error	4	255.8		

The binding curves for glycinin and B-conglycinin are illustrated in Figures 1 to 12 for the three temperatures. It is apparent that saturation was not reached in some cases even at 1000 ppm ligand. At 5°C, only hexane seemed to reach saturation for both proteins. There were quite large deviations from the typical hyperbolic pattern which would be expected if the binding was a simple noninteracting one site type. The errors in the determinations of each binding point were quite large; the coefficients of variation ranged from 1.4 to 12%). This may be due to the difficulties in ensuring the temperature of the water bath, fluctuations in the syringe temperature, the small volume of headspace sampled, errors in injection, etc.

Binding was found for hexane to both glycinin and B-conglycinin at 5°C but not at the higher temperatures. This is not what one would expect; but perhaps structural changes due to the lowered temperature which led to increased ANS hydrophobicity, allowed the interaction with exposed nonpolar groups. This might indicate that an initial non hydrophobic binding is necessary to cause structural changes necessary for hydrophobic binding.

The number of binding sites (Tables 3 and 4) was higher for glycinin than B-conglycinin at all three temperatures. There seemed to be an increase in the number of binding sites at 5°C compared to the higher temperatures for glycinin, but not B-conglycinin. The number of binding sites was greater for the shorter aldehydes at 20° and 30°C, but not 5°C for glycinin. A Wilcoxon signed rank test indicated that the number of binding sites on glycinin were

significantly greater than B-conglycinin at the 0.01 level. A nonparametric test was used because of doubts about the normality of the distribution of the parameters.

Table 3. Number of binding sites for glycinin

Compound	Temperature (°K)		
	278	293	303
Butanal	163 ± 31.7	205 ± 73.2	171 ± 45.5
Pentanal	242 ± 82.0	196 ± 23.9	193 ± 36.4
Hexanal	149 ± 16.1	96 ± 5.8	108 ± 10.2
Octanal	101 ± 4.8	76 ± 5.5	66 ± 3.4
2-Hexanone	165 ± 49.2	58 ± 5.6	67 ± 7.4
3-Hexanone	165 ± 14.3	54 ± 4.8	60 ± 4.5
2-Nonanone	189 ± 32.6	71 ± 3.9	96 ± 14.4
5-Nonanone	245 ± 48.1	67 ± 7.7	65 ± 30.8
hexanol	79 ± 13.0	42 ± 12.0	42 ± 10.1
hexane	14 ± 1.3	NA[a]	NA

[a] no affinity.

Table 4. Number of binding sites for B-conglycinin

Compound	Temperature (°K)		
	278	293	303
Butanal	30 ± 12.6	22 ± 8.1	21 ± 45.5
Pentanal	28 ± 8.7	37 ± 5.9	49 ± 36.4
Hexanal	23 ± 3.9	32 ± 6.5	26 ± 10.2
Octanal	18 ± 2.5	38 ± 5.2	59 ± 3.4
2-Hexanone	23 ± 3.3	24 ± 1.5	38 ± 7.4
3-Hexanone	35 ± 17.5	36 ± 2.7	22 ± 4.5
2-Nonanone	18 ± 1.9	46 ± 42.3	30 ± 14.4
5-Nonanone	19 ± 2.2	44 ± 14	20 ± 30.8
hexanol	12 ± 3.2	8 ± 4.6	10 ± 10.1
hexane	45 ± 11.9	NA[a]	NA

[a] no affinity.

Figure 1. Saturation curves of aldehydes binding to glycinin at 5°C

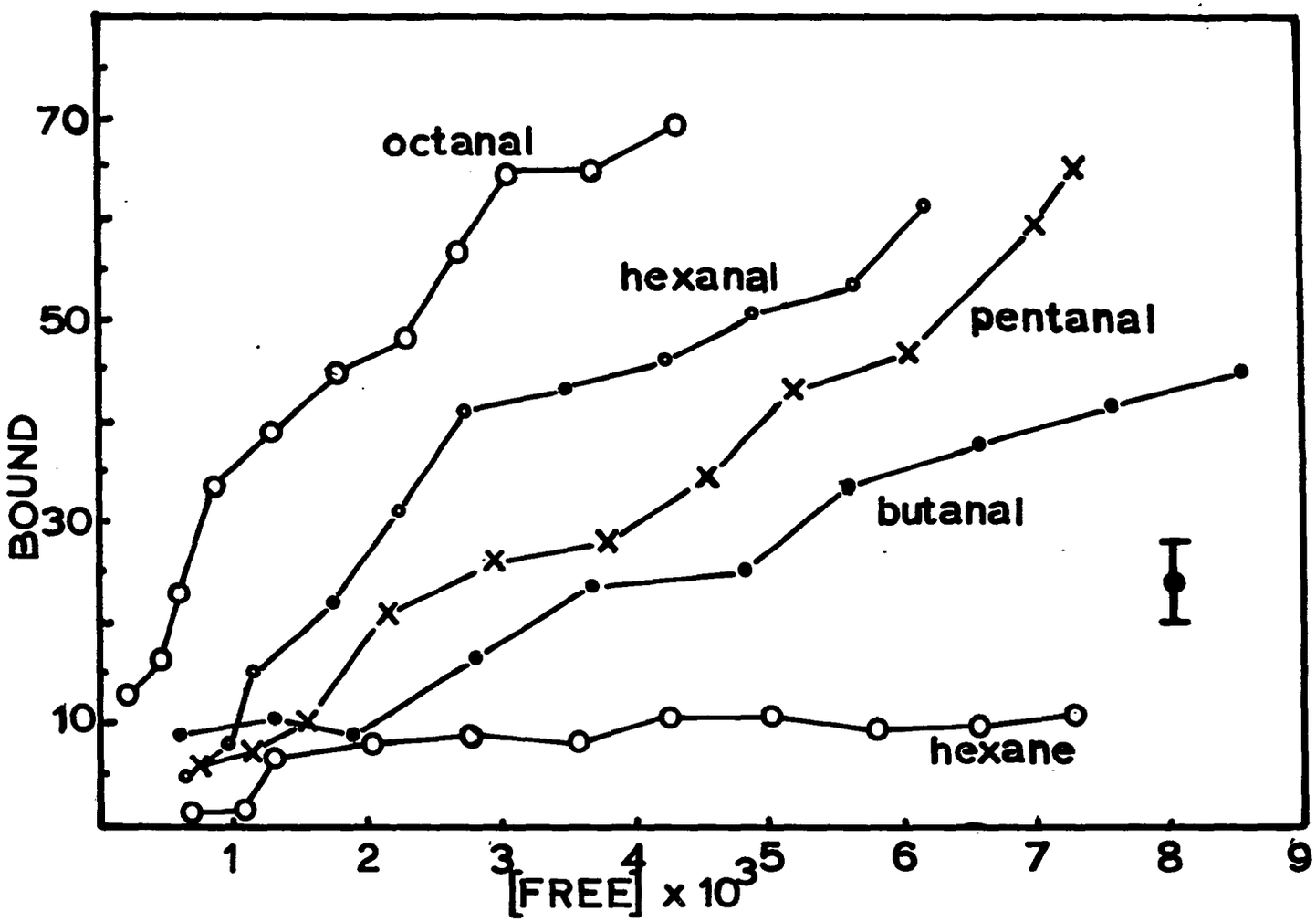

70
50
30
10
BOUND
octanal
hexanal
pentanal
butanal
hexane
1
2
3
4
5
6
7
8
9
[FREE] x 10^3

Figure 2. Saturation curves of ketones, hexanol and hexane binding to glycinin at 5°C

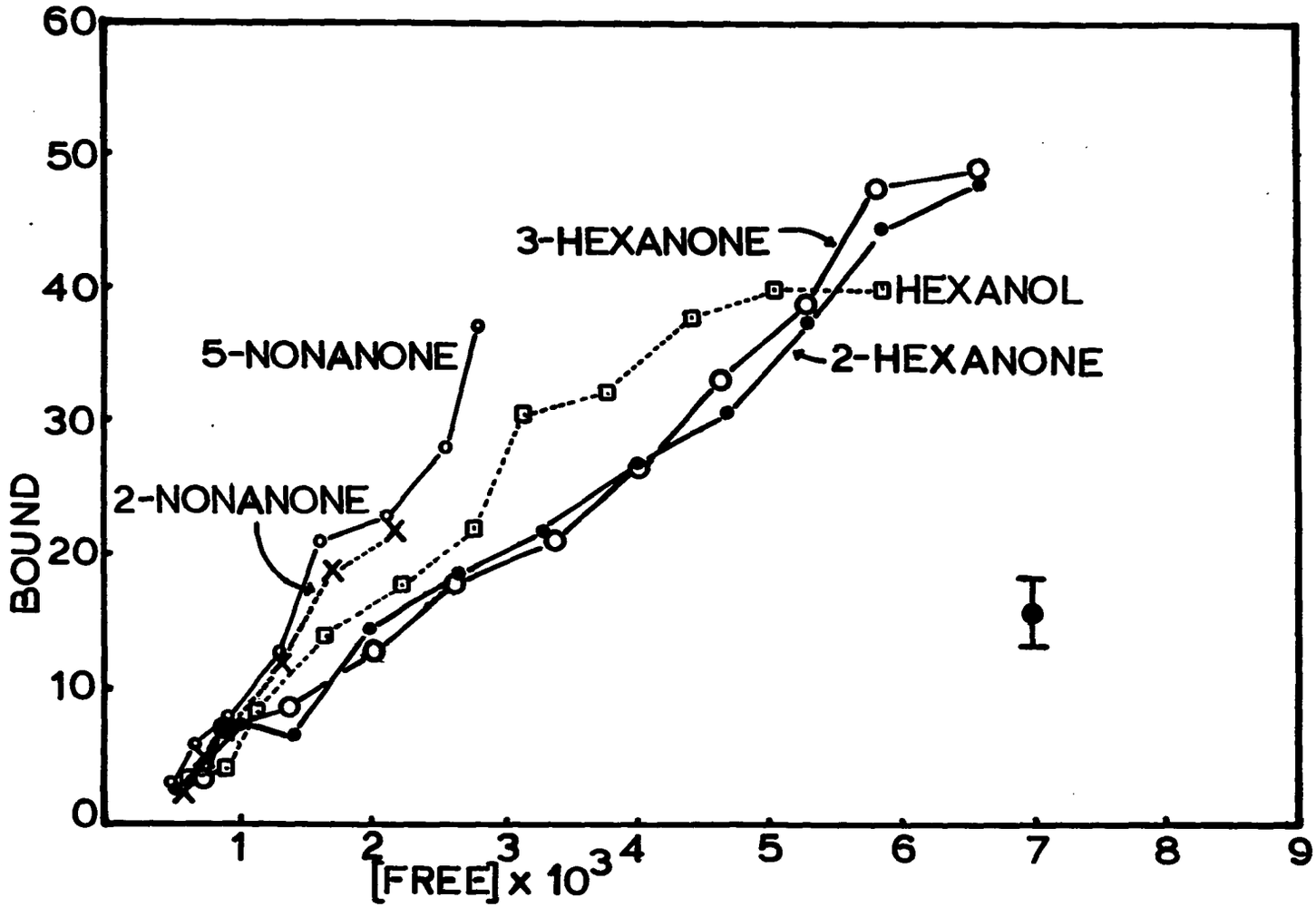

60
50
40
30
20
10
0
BOUND
1
2
3
4
5
6
7
8
9
[FREE] x 10³
3-HEXANONE
HEXANOL
2-HEXANONE
5-NONANONE
2-NONANONE

Figure 3. Saturation curves of aldehydes binding to B-conglycinin at 5°C

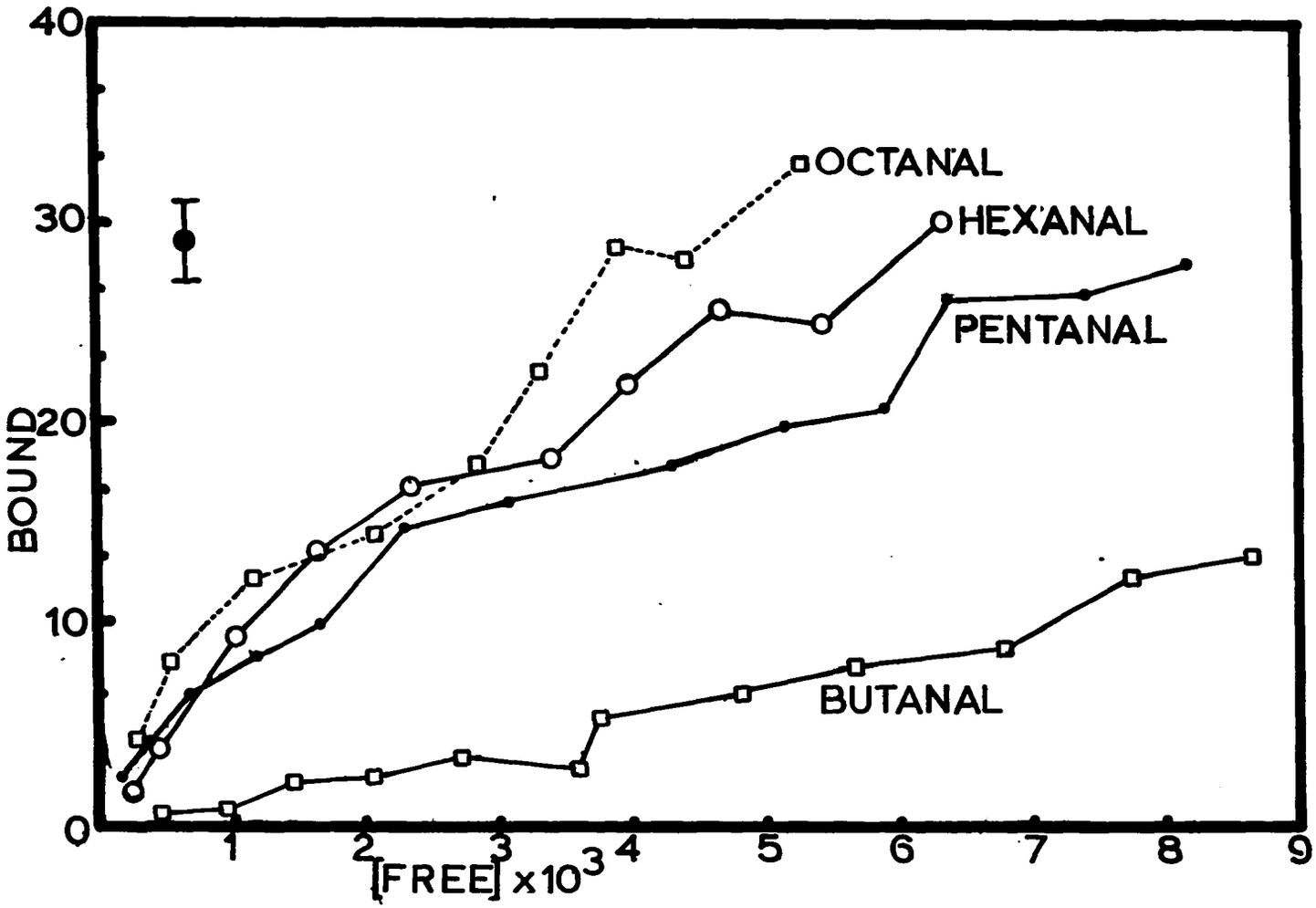

40
30
20
10
0
BOUND
OCTANAL
HEXANAL
PENTANAL
BUTANAL
1
2
3
4
5
6
7
8
9
[FREE] x10^3

Figure 4. Saturation curves of ketones, hexanol and hexane binding to B-conglycinin at 5°C

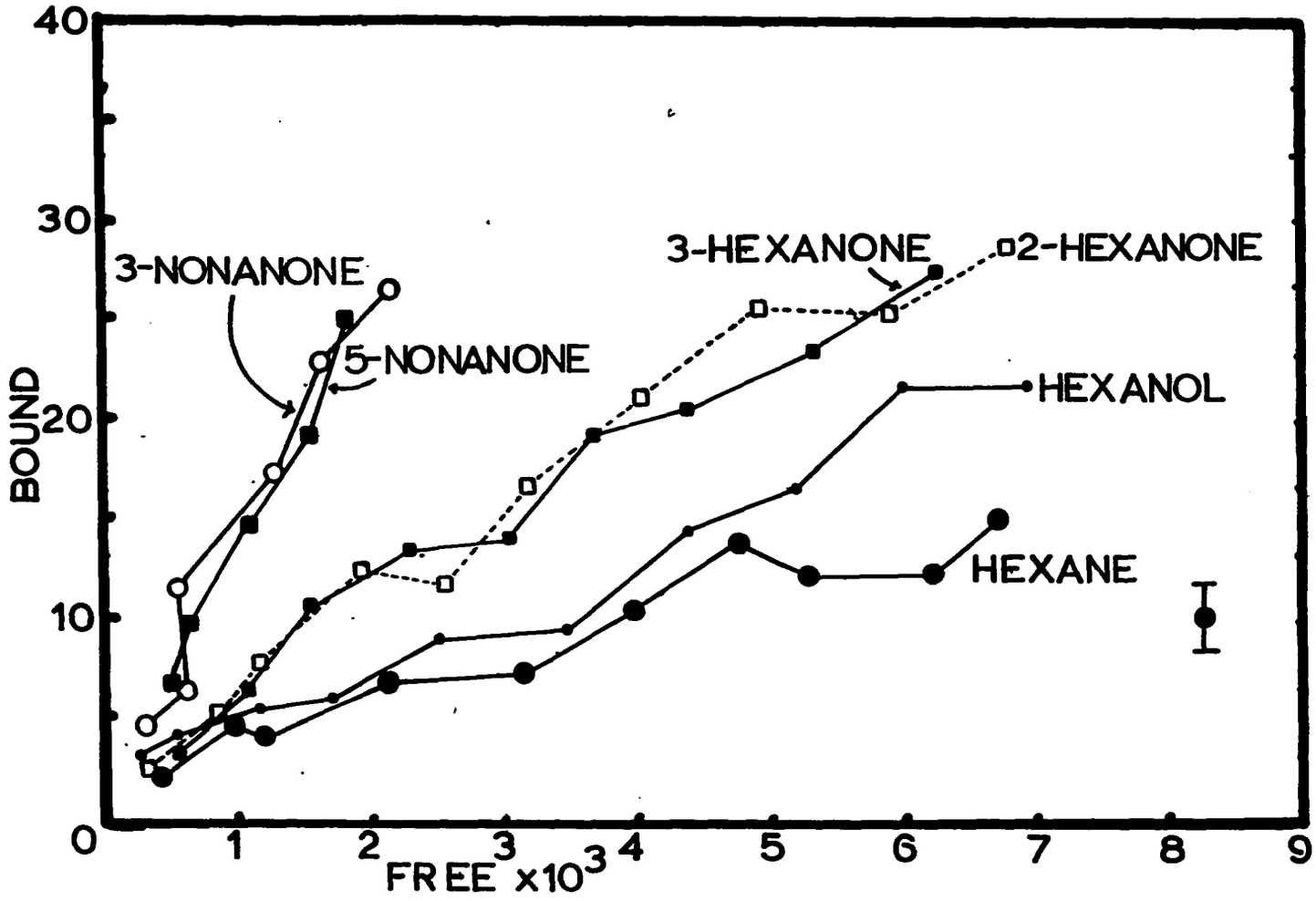

40
30
20
10
0
BOUND
1
2
3
4
5
6
7
8
9
FREE x10³
3-NONANONE
5-NONANONE
3-HEXANONE
2-HEXANONE
HEXANOL
HEXANE

Figure 5. Saturation curves of aldehydes binding to glycinin at $20^{0}C$

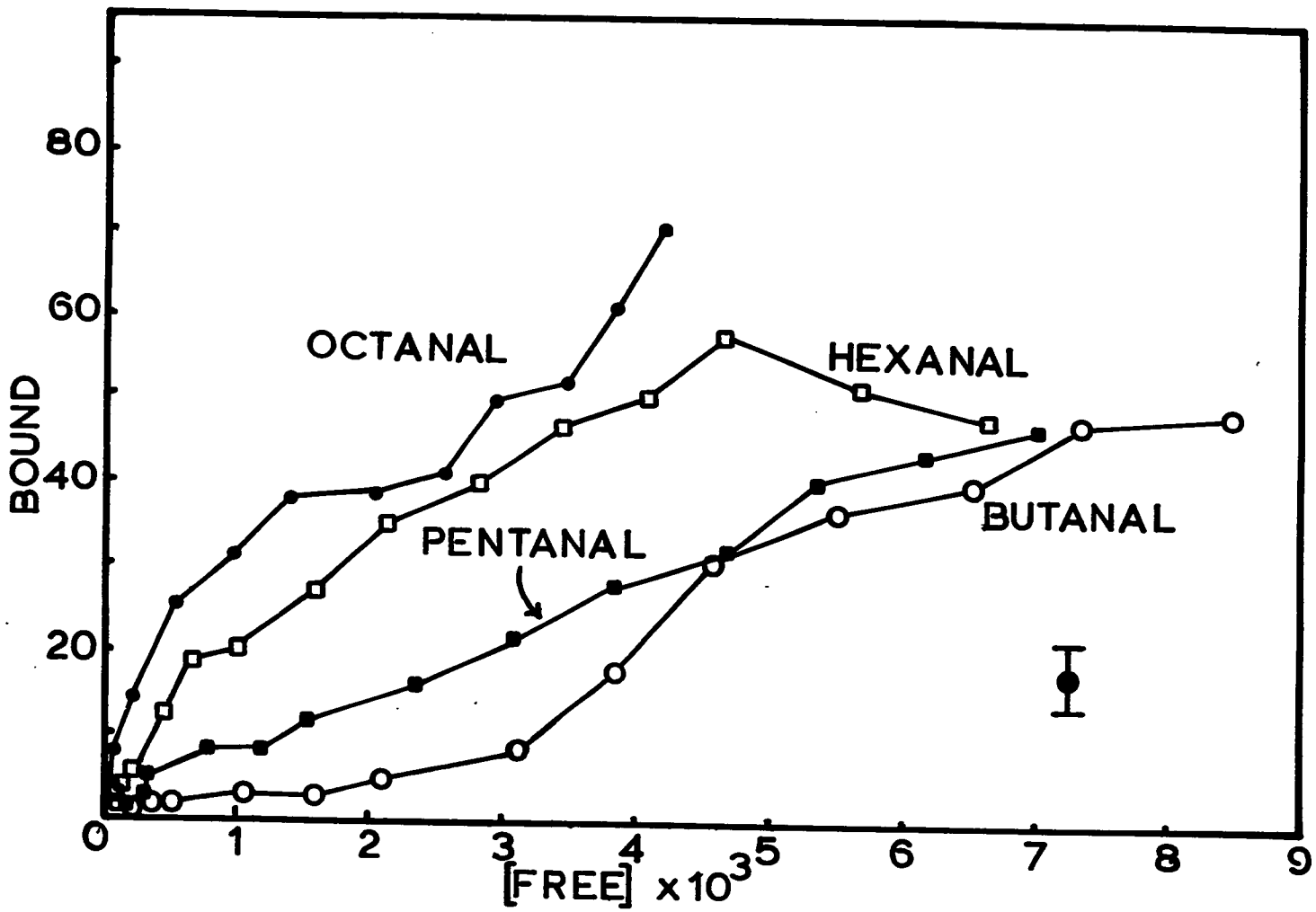

80
60
40
20
0
BOUND
OCTANAL
HEXANAL
PENTANAL
BUTANAL
1
2
3
4
5
6
7
8
9
[FREE] x10³

Figure 6. Saturation curves of ketones, hexanol and hexane binding to glycinin at 20°C

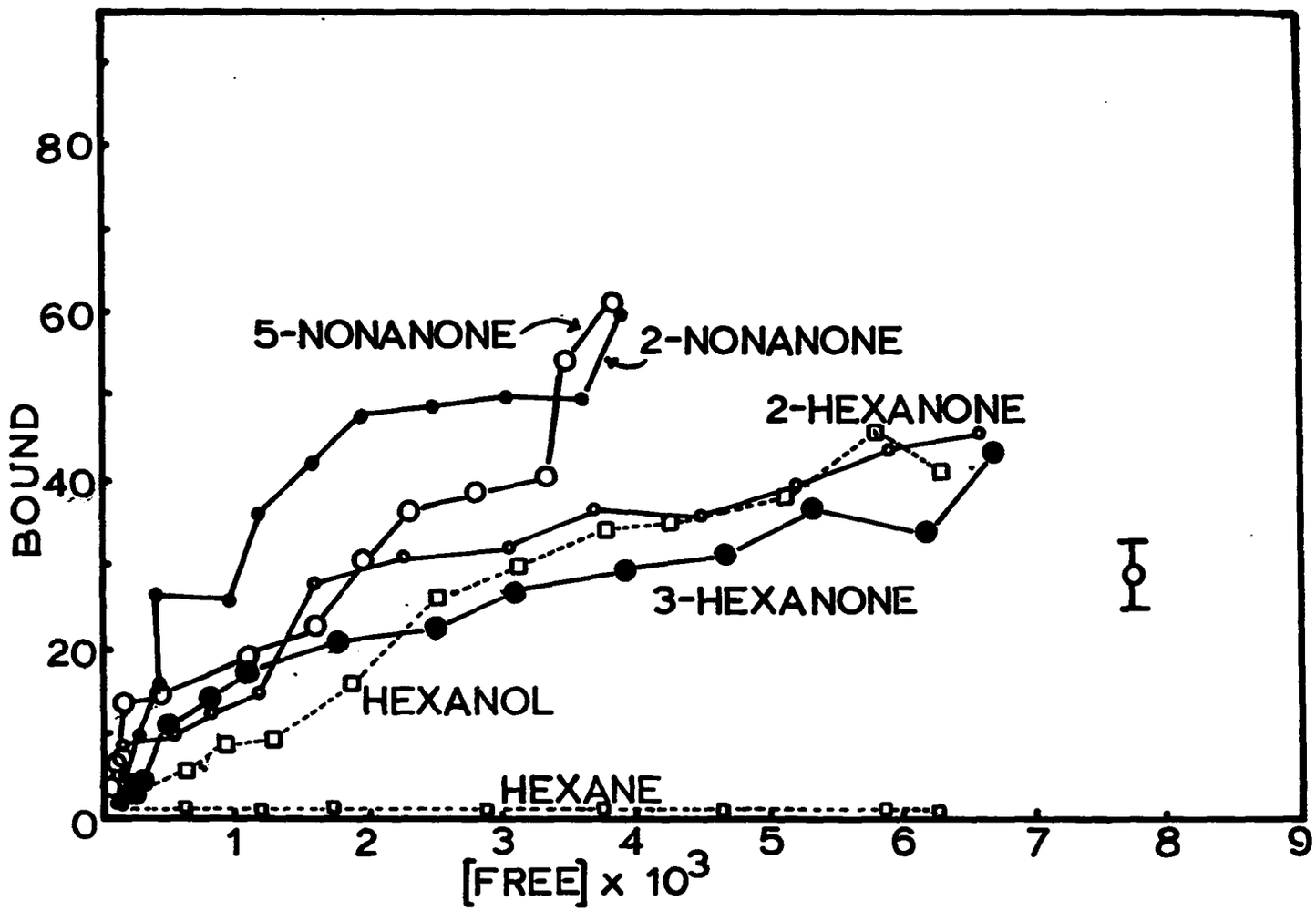

80
60
40
20
0
BOUND
5-NONANONE
2-NONANONE
2-HEXANONE
3-HEXANONE
HEXANOL
HEXANE
1
2
3
4
5
6
7
8
9
[FREE] x 10³

Figure 7. Saturation curves of aldehydes binding to B-conglycinin at 20°C

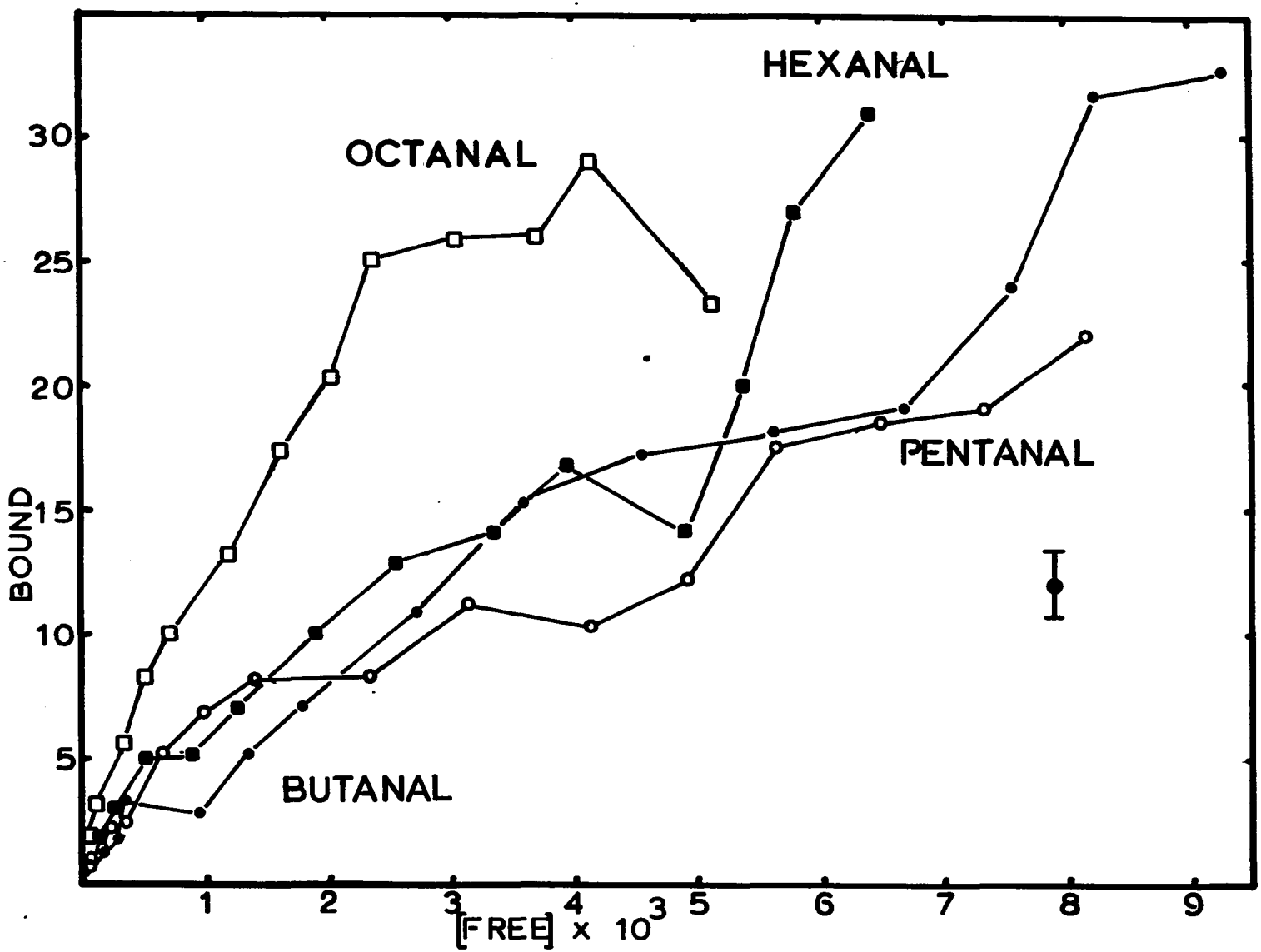

HEXANAL
OCTANAL
PENTANAL
BUTANAL
BOUND
30
25
20
15
10
5
1
2
3
4
5
6
7
8
9
[FREE] x 10^3

Figure 8. Saturation curves of ketones, hexanol and hexane binding to B-conglycinin at 20°C

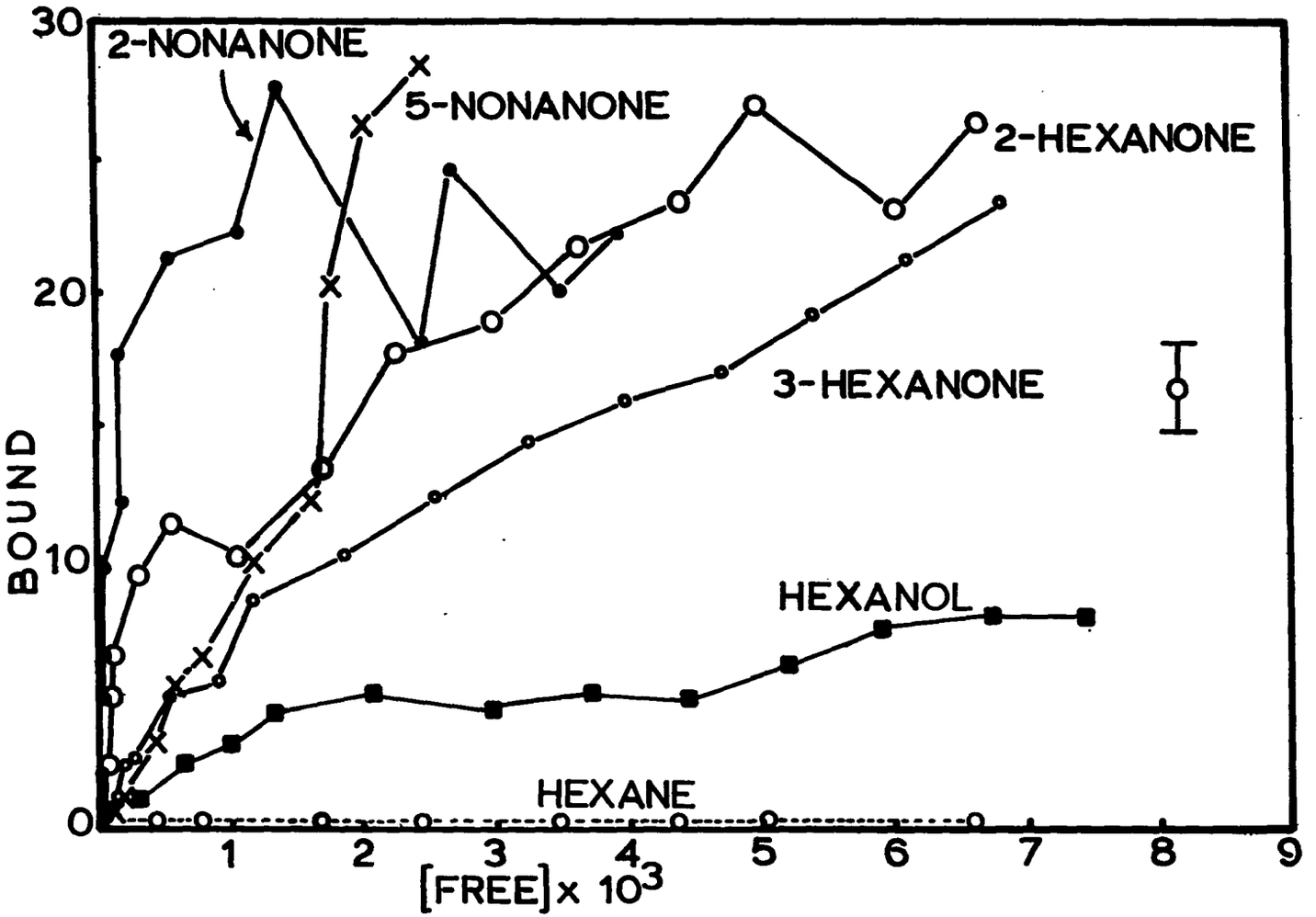

30
20
10
0
BOUND
2-NONANONE
5-NONANONE
2-HEXANONE
3-HEXANONE
HEXANOL
HEXANE
1
2
3
4
5
6
7
8
9
[FREE] x 10³

Figure 9. Saturation curves of aldehydes binding to glycinin at 30°C

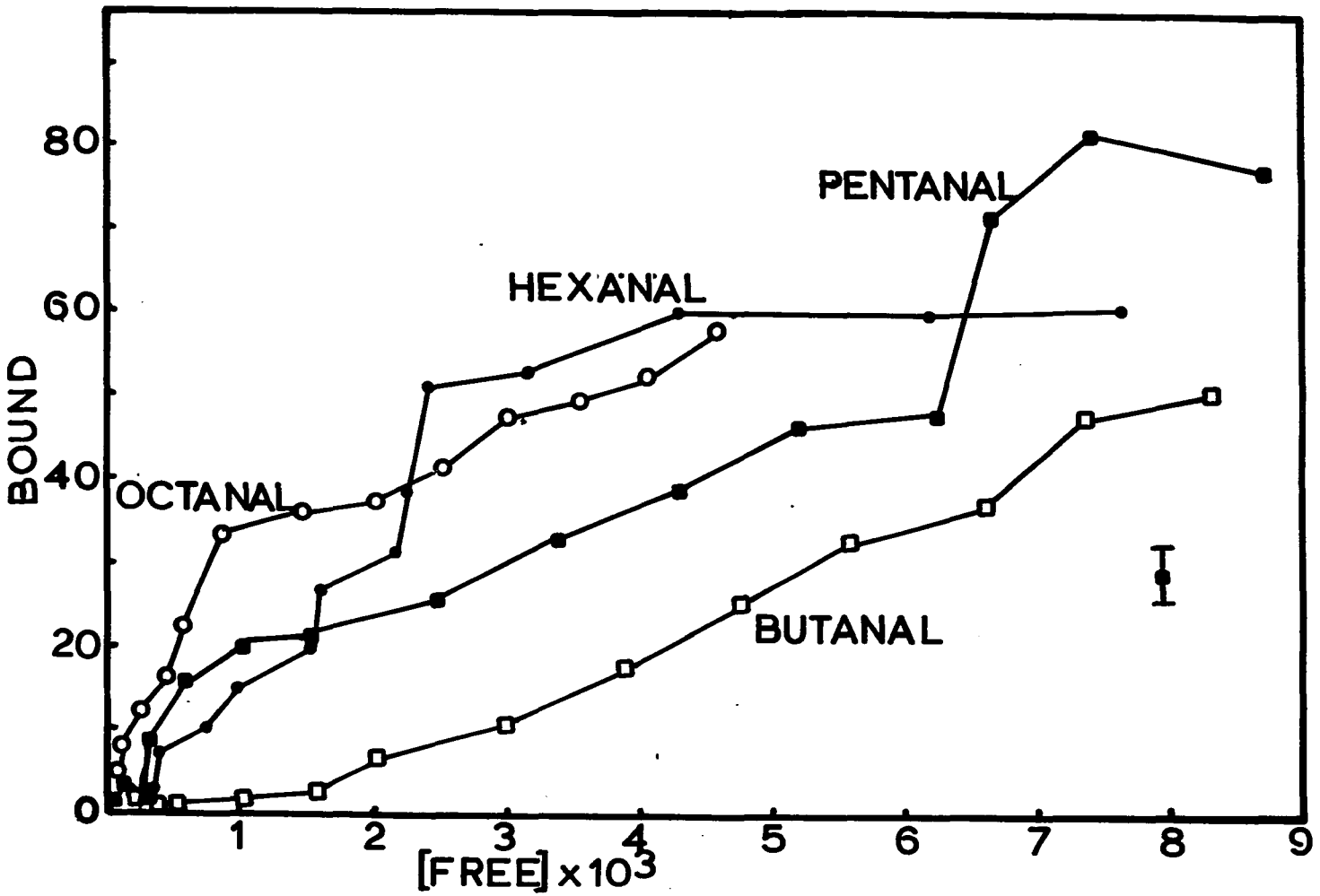

80
60
40
20
0
BOUND
PENTANAL
HEXANAL
OCTANAL
BUTANAL
1
2
3
4
5
6
7
8
9
[FREE] x10³

Figure 10. Saturation curves of ketones, hexanol and hexane binding to glycinin at 30°C

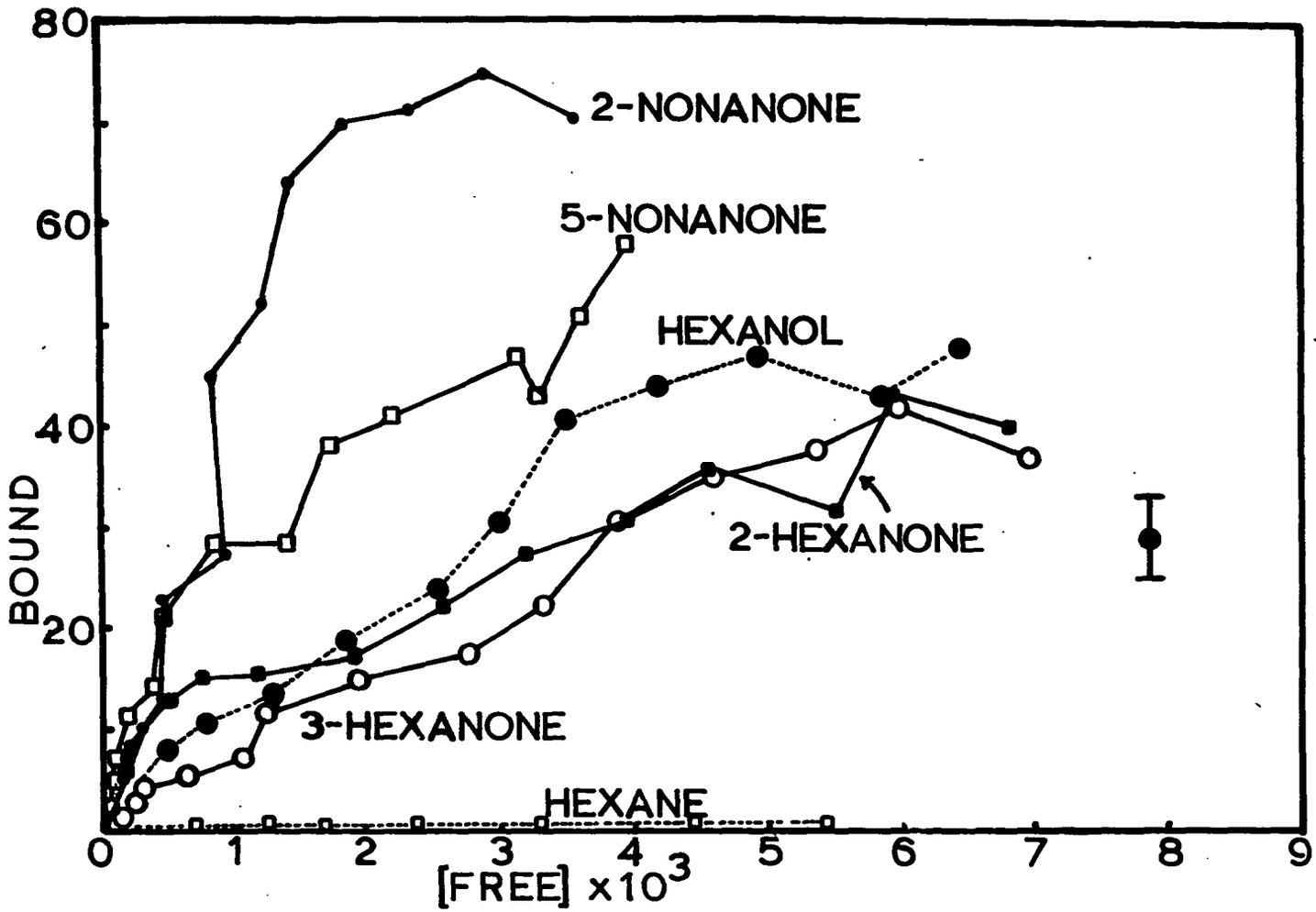

80
60
40
20
BOUND
2-NONANONE
5-NONANONE
HEXANOL
2-HEXANONE
3-HEXANONE
HEXANE
0
1
2
3
4
5
6
7
8
9
[FREE] x10³

Figure 11. Saturation curves of aldehydes binding to B-conglycinin at 30°C

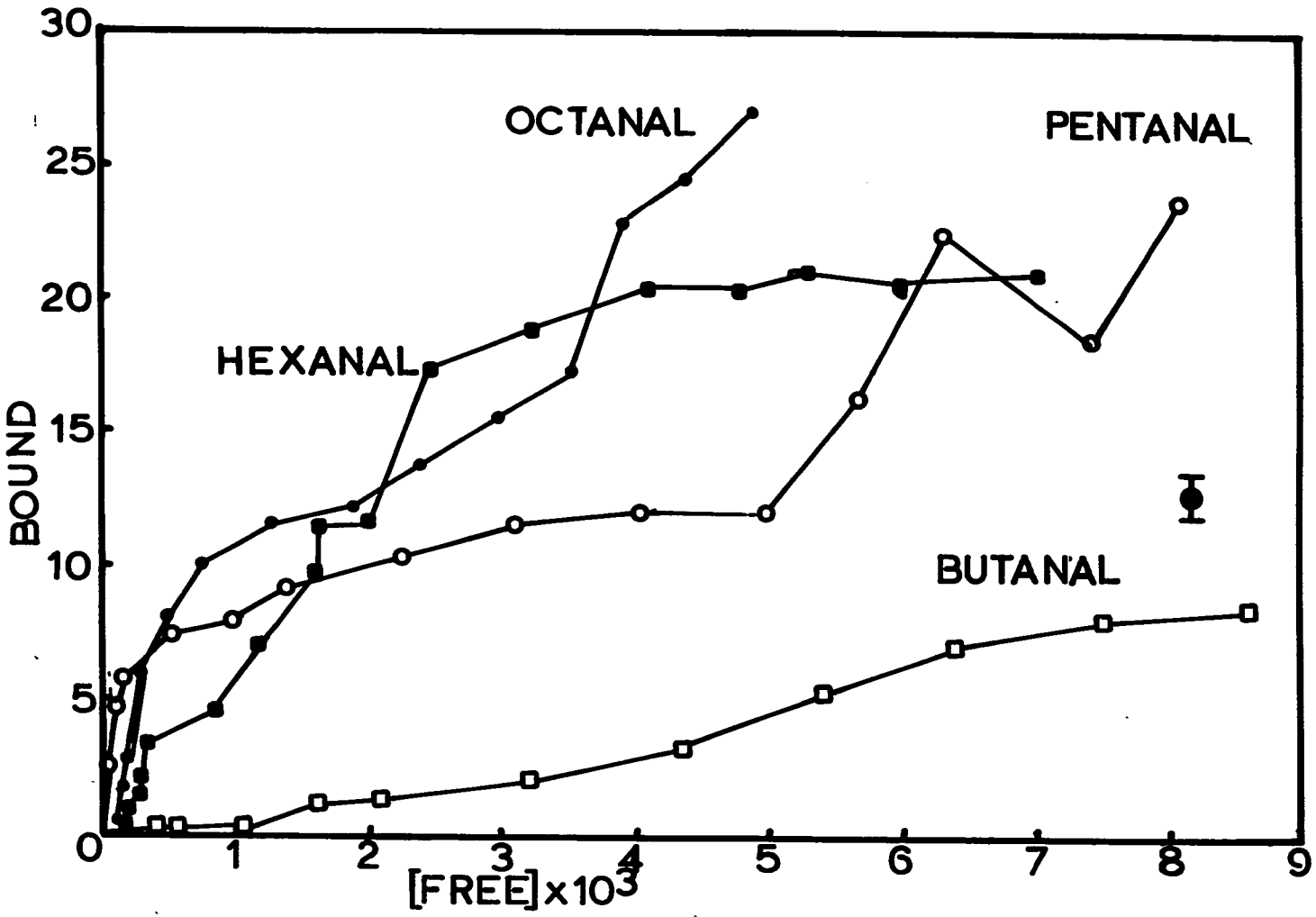
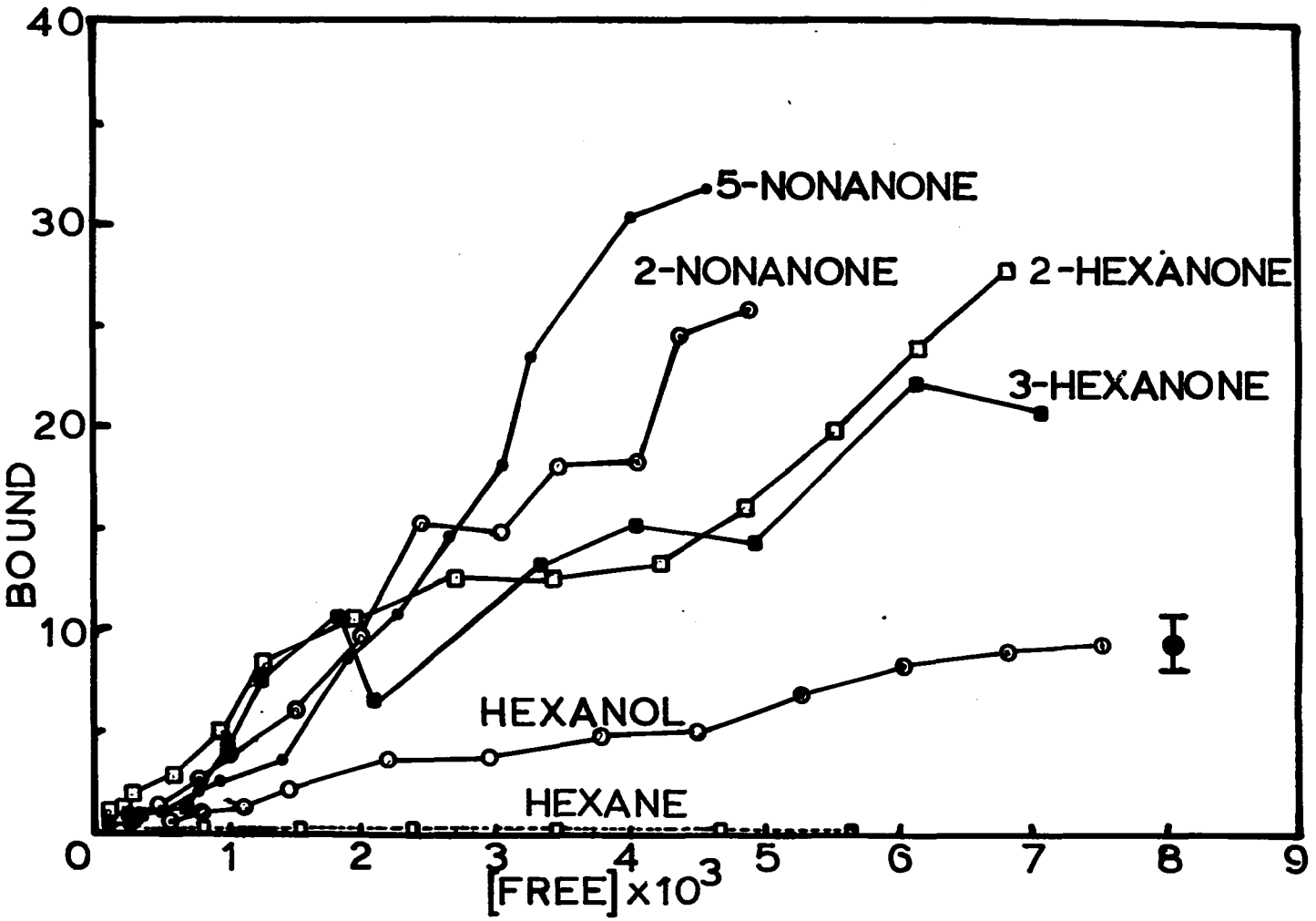

30
25
20
15
10
5
0
BOUND
OCTANAL
PENTANAL
HEXANAL
BUTANAL
1
2
3
4
5
6
7
8
9
[FREE]x10³

Figure 12. Saturation curves of ketones, hexanol and hexane binding to B-conglycinin at 30°C


40
30
20
10
0
BOUND
5-NONANONE
2-NONANONE
2-HEXANONE
3-HEXANONE
HEXANOL
HEXANE
1
2
3
4
5
6
7
8
9
[FREE] x10^3

The high errors are apparently due to the variations in the binding data due to the precision limitations of the equilibrium headspace sampling system used but also the fact that the binding observed did not fit the binding model. Unfortunately, the reporting of errors from regression determined data is often omitted, so it is difficult to compare precisions of the estimated parameters. There did not seem to be an effect of temperature on the number of sites for either protein for the aldehydes. The number of sites on glycinin but not B-conglycinin was higher at 5°C for the ketones, hexanol, and hexane. This agrees with the lack of saturation observed at 5°C compared to the higher temperatures. The lack of saturation even at 1000 ppm illustrates the high binding capacity of the soy proteins. The reason for the much differences in the number of sites for hexane binding to glycinin or B-conglycinin compared to the other ligands is unknown. The number of sites for hexane was higher than the other ligands for B-conglycinin but lower for glycinin. The number of sites was lower for hexanol compared to hexanal and 2-hexanone, for both proteins.

The binding constants (Tables 5 and 6) increased as chain length increased for the aldehydes. A Wilcoxon signed rank test indicated that the binding constants were higher for glycinin at 20° and 30°C ($p<0.01$, 0.02, respectively) but not 5°C. The change in the G indicated that the energy increased from 230 to 532 cal/CH_2 as the chain length increased (Table 7). The increase in energy was greater at higher temperatures for B-conglycinin whereas it was lower for

Table 5. Equilibrium binding constants for glycinin

Compound	Temperature (°K)		
	278	293	303
Butanal	14 ± 21.2	39 ± 33.6	44 ± 12.9
Pentanal	40 ± 20.9	87 ± 22.9	119 ± 37.2
Hexanal	116 ± 17.1	270 ± 24.1	455 ± 101.0
Octanal	600 ± 55.6	833 ± 75.6	902 ± 90.9
2-Hexanone	244 ± 50.2	411 ± 41.1	500 ± 166.7
3-Hexanone	255 ± 33.2	301 ± 55.1	357 ± 77.6
2-Nonanone	588 ± 126.0	891 ± 42.3	1000 ± 111.1
5-Nonanone	661 ± 191.3	714 ± 194.8	769 ± 188.0
hexanol	260 ± 39.9	470 ± 44.1	344 ± 52.6
hexane	556 ± 111.0	NA[a]	NA

[a] no affinity.

Table 6. Equilibrium binding constants for B-conglycinin

Compound	Temperature (°K)		
	278	293	303
Butanal	32 ± 16.1	21 ± 9.8	18 ± 6.6
Pentanal	107 ± 31.0	67 ± 38.1	149 ± 43
Hexanal	171 ± 24.7	256 ± 160.3	303 ± 30.2
Octanal	201 ± 47.6	200 ± 63.2	588 ± 320.9
2-Hexanone	251 ± 13.3	385 ± 170.9	476 ± 111.0
3-Hexanone	131 ± 40.0	94 ± 30.0	217 ± 32.6
2-Nonanone	226 ± 74.0	254 ± 10.9	303 ± 66.0
5-Nonanone	332 ± 83.2	326 ± 6.4	396 ± 120.7
hexanol	192 ± 126.5	181 ± 16.2	131 ± 23.9
hexane	541 ± 189.3	NA[a]	NA

[a] no affinity.

Table 7. Change in free energy of binding per CH_2 in homologous series of aldehydes (cal/CH_2)

Protein	Temperature (°K)		
	278	293	303
Glycinin	532	460	457
B-conglycinin	230	322	480

Table 8. Thermodynamic parameters for the interaction of carbonyls with soy glycinin and B-conglycinin

Protein Compound	ΔH^o Kcal/mol	ΔG^o Kcal/mol			ΔS^o cal/mol°K		
		Temperature (K)					
		278	293	303	278	293	303
Glycinin							
Butanal	8.00	-1.46	-2.14	-2.28	34.0	34.6	33.9
Pentanal	7.71	-2.04	-2.60	-2.88	35.1	35.2	35.0
Hexanal	9.16	-2.63	-3.26	-3.69	42.4	42.4	42.4
Octanal	2.81	-3.54	-3.92	-3.51	22.8	23.0	20.9
2-Hexanone	4.89	-3.04	-3.51	-3.75	28.5	28.7	28.5
3-Hexanone	2.21	-3.06	-3.33	-3.54	19.0	18.9	19.0
2-Nonanone	3.65	-3.53	-3.96	-4.16	25.8	26.0	25.8
5-Nonanone	0.99	-3.59	-3.83	-4.00	16.5	16.5	15.9
Hexanol	2.32	-3.07	-3.59	-3.52	19.4	20.2	19.3
Hexane	-	-3.50	-	-	-		
B-conglycinin							
Butanal	-3.92	-1.91	-1.77	-1.74	-7.2	-7.3	-7.2
Pentanal	1.51	-2.58	-2.45	-3.02	14.7	13.5	15.0
Hexanal	3.89	-2.84	-3.23	-3.44	24.2	16.2	16.3
Octanal	6.49	-2.93	-3.09	-3.84	33.9	32.7	34.1
2-Hexanone	4.32	-3.06	-3.47	-3.72	26.5	26.6	26.5
3-Hexanone	2.70	-2.69	-2.65	-3.24	19.4	18.3	19.6
2-Nonanone	1.89	-3.00	-3.23	-3.44	17.6	17.5	17.6
5-Nonanone	1.05	-3.21	-3.37	-3.60	15.3	15.1	15.3
Hexanol	-2.37	-2.91	-3.03	-2.94	1.9	2.3	1.9
Hexane	-	-3.48	-	-	-		

glycinin. These data are lower than reported by Damodaran and Kinsella (1981a) who found an increase of 600 cal/CH_2 for ketone interaction with soy protein isolate at 25°C. Aspelund and Wilson (1983) found an increase by 578 cal/CH_2 in a dry model system where hydrophobic binding would not take place. Damodaran and Kinsella (1981a) indicated that this range of change has been reported for hydrophobic associations, but this does not explain why a similar value was obtained in the dry system, unless the van der Waals and hydrophobic forces have equal magnitudes.

The thermodynamic data (Table 8) indicate that the $T\Delta S$ was greater than ΔH; the entropy was driving the reaction. This was previously reported by Thissen for soy isolate. It is, however, difficult to clearly establish, from the data presented alone, what type of binding is occurring. There may be a number of interrelated types of binding and structural changes occurring at the same time. The assumptions, such as a true equilibrium existing, which were necessary for our study may not be true. It would be an overoptimistic simplification to state that the binding is hydrophobic because the free energy increased as the chain length of the aldehydes increased.

Conclusions

There were limitations in the headspace technique at low and high temperatures. The hydrophobicity data indicated that there were changes in the binding of the hydrophobic probe at 5° compared to

20°C. This could be interpreted in terms of a structural change either increasing the strength or number of binding sites or possibly changes in fluorescent yield. The number of binding sites was higher at 5°C for glycinin than at 20° or 30°C, suggesting a greater number of binding sites for the ANS molecule. However, Hawakawa and Nakai (1985) indicate that the binding sites for ANS and the aliphatic probe, cis-paranaric acid, may be different. It is possible for structural rearrangement to change both the number and strength of the binding sites for the hydrophobic probes and the flavor compounds. Thus, interpretation would be difficult unless the parameters were separated.

The numbers of binding sites were higher for glycinin at all temperatures studied and the binding constants were higher for glycinin at 20° and 30°C. The numbers of binding sites on both proteins were higher than previously reported and the binding constants were higher for glycinin than B-conglycinin, in disagreement with the work by Damodaran and Kinsella (1981). The reasons for these differences may include the differences in the model systems used (sodium azide, 2-mercaptoethanol). Further study is necessary to understand the events that occur during soy protein-flavor compound interactions.

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SUMMARY AND DISCUSSION

The numbers of binding sites for all of the carbonyl compounds studied were greater for glycinin than B-conglycinin. The number of binding sites was greater, for glycinin but not B-conglycinin, at 5°C compared to the higher temperatures. The binding constants were more variable but were higher for glycinin than B-conglycinin at 20° and 30°C. These findings do not agree with the reports of Kinsella and coworkers. The reasons for the differences probably include the differences in binding behavior in the presence of sodium azide and 2-mercaptoethanol as well as the difficulties in interpreting partial binding curves. Sodium chloride (0.5M) affected the binding but it is difficult to say, from the experiments performed here, whether the differences were due to structural changes in the protein or an effect on the binding itself. Smaller changes in salt that would not affect the structure so drastically may differentiate these possibilities.

The reasons for the high error terms in the parameters determined by the Wilkinson procedure probably include the fact that the simple binding model is not sufficient for complicated binding phenomena. A stepwise computer program is probably necessary but the number of data points and replications per point would have to be increased from those used in this study to increase the confidence in the data. A headspace model system which involves greater than the 100ul gas sampling which was used in this study may decrease some of the errors but more protein would be necessary. The report of highly pure

protein production from a combination of isoelectric precipitation and ammonium sulfate fractionation (Iwabuchi and Yamauchi, 1987) may allow rapid production of sufficient protein to use a scaled up model system where errors might be smaller.

The effect of the small amounts of lipid on binding would be an interesting area of research. Also, vacuum treatment with reagents known to interfere with different types of binding may give more specific information than what was presented here. One line that may provide useful results would be to use an equilibrium dialysis with ^{14}C ligand, determine the total bound by the radioactivity in the two sides at equilibrium, extract the protein side with reagents which would remove all noncovalently bound ligand, and thus determine the amount of bound ligand which could not be removed by solvent extraction (covalent ?). This would give an estimate of covalent and noncovalent binding in the same sample and the use of radioactive ligand would allow a high degree of sensitivity. The equilibrium dialysis procedure would likely be much less temperature sensitive. It is probably important to add the ligand in an aqueous solution to avoid problems of differences in the rates of solubilization of the different ligands.

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