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Bound and free water relationships in soy proteins as measured by differential scanning calorimetry

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by

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INTRODUCTION

Water is the major component of most foods. Its presence in the proper location, amount, and orientation is necessary for acceptable food quality, particularly texture (Fennema, 1976). Many methods of food preservation are based on changes in the aqueous part of food systems. Freezing is a common example of this. Water activity (a_w) exerts a major influence on the microbial, chemical, and enzymatic stability of foods.

Water activity is proportional to the vapor pressure exerted by the water in a food and is a measure of the effective concentration of water as a reactant in chemical reactions (Bone, 1973). This effective concentration is determined by various types of restraint imposed on the behavior of the water (Desrosier and Desrosier, 1977). These restraints are mediated by many systemic factors such as: the nature and concentration of dissolved components; the water binding capacity and number of polar residues; the configuration of hydrophobic and hydrophilic areas; and presumably, the mechanisms which alter the structure of water itself (Brockman, 1970).

Alteration of water activity to prevent bacterial growth while retaining a soft-moist texture is the basis for production of intermediate moisture foods. One or

more humectants are usually added to reduce the a_w. Humectants used most frequently are polar or ionic solutes such as sucrose, sorbitol, glycerol, sodium chloride, and propylene glycol (Sloan et al., 1977).

When a solute is added to water, the concentration of water is reduced, which reduces its vapor pressure. The solute may also break or increase the water structure. Sucrose, for example, appears to create more structure in the water, thus reducing the effective water concentration below its actual concentration (Bone, 1973).

The ideal humectant for human foods has not been found. Those few which are effective in lowering a_w have other disadvantages which make them undesirable. Perhaps if more were known about water binding and the effect of solutes on water structure, this would aid the development of a_w control technology.

The water surrounding a nonpolar solute becomes more structured and as a result of this increased ordering, the effective concentration of the water is reduced (Bone, 1973). The resultant a_w reduction is proportional to the molecular weight of the nonpolar solute (Bone, 1969). For example, polyethylene glycol lowers a_w much more than predicted by its concentration, and this effect increases tremendously as its molecular weight increases (Bone, 1969).

Proteins can also affect a_w in a way not predicted

by their effect on osmotic pressure (concentration). The addition of a small amount of caseinate to a food system containing low-molecular weight solutes lowers the a_w much more than accounted for by reduction of water concentration (Bone, 1969). Bone et al.(1975) found that a lower a_w was obtained in a food system when collagen was added before sucrose and allowed to hydrate first without the competition of sucrose, rather than the reverse sequence with sucrose added first. These protein effects have not been explained, but they may be important.

Information about water binding is also necessary and helpful to the development of food dehydration processes. Dehydration can be difficult and expensive when the food components bind substantial quantities of water (Berlin et al., 1973). With the prospect of energy shortages, it is becoming increasingly important for scientists to obtain thermodynamic data that can aid in the development of efficient dehydration techniques (Berlin and Kliman, 1974).

In this study, the water binding properties of various soy protein products and ovalbumin were studied. The information gained and the conclusions drawn should apply not just to these proteins, but to all food proteins. This type of information should be useful in the development of water activity control and dehydration technology.

LITERATURE REVIEW

Water

Molecular chemistry

A water molecule is formed when two hydrogen atoms approach the two sp^3 bonding orbitals of oxygen and form two covalent sigma bonds that have 40 percent ionic character. The angle between the oxygen and hydrogen atoms is 105 degrees. The two negative and two positive charges form the angles of a regular tetrahedron. The resultant V-like shape of the water molecule as well as the polarity of the oxygen-hydrogen bond itself result in an asymmetrical charge distribution. Molecular polarity of this magnitude leads to intermolecular attractive forces, which result in three-dimensional hydrogen bonding between water molecules (Fennema, 1976).

The highly electronegative oxygen can be visualized as partially pulling away the electrons from the two covalently bonded hydrogen atoms, thereby leaving each hydrogen atom with a partial positive charge. The hydrogen-bonding orbitals of the hydrogen atoms are located on two of the axes of the imaginary tetrahedron. These two axes can be thought of as representing lines of positive force (hydrogen bond donor sites). The oxygen atom's two

free orbitals are located on the remaining two axes of the imaginary tetrahedron and can be thought as of representing lines of negative force (hydrogen bond acceptor sites) (Fennema, 1976).

The existence of these four lines of force means that each water molecule can potentially hydrogen bond with four others. Because water molecules have an equal number of hydrogen bond donor and receptor sites arranged to allow three-dimensional hydrogen bonding, the attractive forces among water molecules are very great relative to the attractive forces among other small molecules that participate in hydrogen bonding. The reason for this is that all of the potential hydrogen bonding sites in water can be satisfied, whereas they can't be in something like NH_3 which has unequal numbers of donor and receptor sites (Fennema, 1976).

Cooperative hydrogen bonding

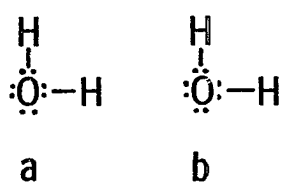
When hydrogen bonding is assessed from a quantum point of view, the contribution made by the different resonance configurations of water molecules can be examined (Drost-Hansen, 1966). So-called exchange forces between water molecules generated by changes in their resonance configuration can contribute to the strength of the hydrogen bond and hence, to the overall stability and structure of liquid water.

This idea is illustrated in Figure 1. When a hydrogen bond forms between the resonance states designated by "a" and "b", these resonance forms are stabilized. One of the original molecules is more negative and the other more positive than each was originally. The covalent bond, in other words, takes on a more nearly ionic character. The original bond generates more bonds because the bonded molecules now bind more easily and more strongly to other molecules than they initially did to each other. This concept is called cooperative hydrogen bonding. Frank and Quist (1961) have argued that such a resonance-induced charge separation mechanism might lead to cooperative behavior of the whole hydrogen bonding phenomenon on a grand scale, resulting in large aggregates, or clusters, of molecules.

Water structure

The idea that liquid water has structure is widely accepted (Drost-Hansen, 1966). There are numerous concepts of the nature of this structure, but they fall into two broad categories. Uniform, or continuum, theories of water assume that intermolecular hydrogen bonds are distributed uniformly so that each water molecule has essentially the same environment (Kell, 1972). In contrast, mixture theories of water structure assume that intermolecular

Figure 1. Resonance in hydrogen-bonded water molecules leading to cooperative hydrogen bonding. Taken from Drost-Hansen (1966).



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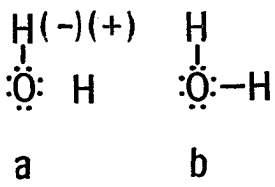

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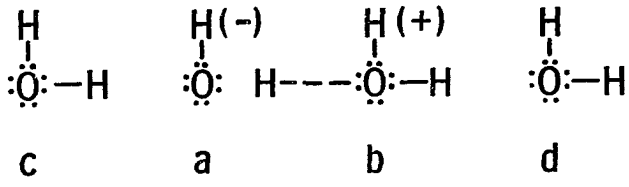


III

bonds, at any given moment, are localized in multi-molecular clumps, or clusters, of water molecules (Davis and Jarzynski, 1972). The properties of liquid water are better explained by the mixture models than by the uniform models (Drost-Hansen, 1966).

One mixture model which satisfactorily explains many properties of water has been proposed by Frank and Quist (1961). They suggest that the formation of a bonded structure in liquid water is a cooperative process in which flickering clusters of varying extent form, relax, and reform in a temporal sequence and spatial pattern determined by the energy fluctuations that are constantly occurring. Some fraction of the water exists as unbonded, monomeric molecules that occupy interstitial sites in a bonded framework. The model requires the existence of an equilibrium between the different forms of water which can be shifted by various factors.

One way to shift this equilibrium in the direction of greater structure is the addition of nonpolar solutes (Frank and Quist, 1961). Nonpolar solutes such as methane decrease the entropy of a solution. Nonpolar amino acids such as valine, methionine, isoleucine, and leucine have the same effect. Frank and Quist (1961) believe this entropy decrease results from some of the monomeric water forming or joining structured water clusters.

Other workers, while confirming the entropy decrease that results from addition of nonpolar functional groups, explain the water structure that forms in a different way. Klotz (1958) suggests that nonpolar side chains in proteins induce a cagelike arrangement of hydration water around the nonpolar groups, with the possibility of long-range cooperative bonding due to the presence of many such nonpolar side chains on the protein molecule. Indeed, approximately 41 percent of protein amino acids have nonpolar side chains. Klotz used this concept to explain various phenomena of proteins in solution such as the masking of functional groups, denaturation, apparent volume changes, and cooperative effects in binding of small molecules to proteins. This idea of nonpolar groups being held in a "cage" of water is similar to the structure of the well-known clathrate hydrates of low molecular weight hydrocarbons and halogen gases in which the molecules are trapped inside a water lattice (Drost-Hansen, 1966).

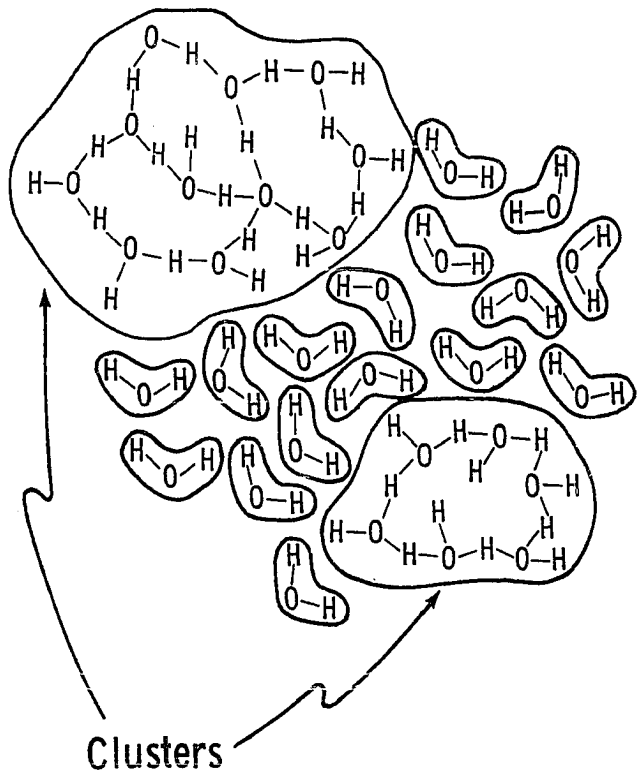
The idea of water around nonpolar groups shifting to a clathrate-type structure is related to Pauling's (1960) concept of pure liquid water being a clathrate hydrate of itself. In this model, water forms pentagonal (Berendsen, 1967) clathrate cages, and the enclosed sites are occupied by unbonded water molecules. In Klotz's model, these unbonded water molecules are replaced by nonpolar solutes.

Pauling's model is considered by many to be too ordered and rigid for a liquid of low viscosity (Frank and Quist, 1961; Nemethy and Scheraga 1962a; Drost-Hansen, 1966). Frank and Quist's "flickering" clusters, which form and breakdown about 10^{11} times per second, introduce the necessary fluidity into the model. The frequency with which the molecules vibrate around their equilibrium positions in the clusters is much higher: 10^{13}-10^{14} times per second.

A mixture theory for liquid water which has many similarities to Frank and Quist's model has been described by Nemethy and Scheraga (1962a). This theory has achieved the greatest success in predicting the thermodynamic properties of water and the effect of nonpolar solutes on those properties (Drost-Hansen, 1966). Nemethy and Scherago accept the idea of resonance in hydrogen-bonded water molecules and the formation of hydrogen bonds being a cooperative process leading to the formation of short-lived water clusters. Their concept of clusters is shown in Figure 2.

The clusters are considered to be imbedded in and in equilibrium with monomeric non-hydrogen bonded water. Hydrogen bonds do not exist between the monomeric molecules, but these molecules do participate in dipole-dipole and London interactions with neighboring molecules. Depending on conditions, the clusters may grow by attachment of

Figure 2. Schematic representation of a model of water showing hydrogen bonded clusters and unbonded molecules. The molecules in the interior of the clusters are tetra-coordinated, but not drawn as such in this two-dimensional diagram. Taken from Nemethy and Scheraga (1962a).

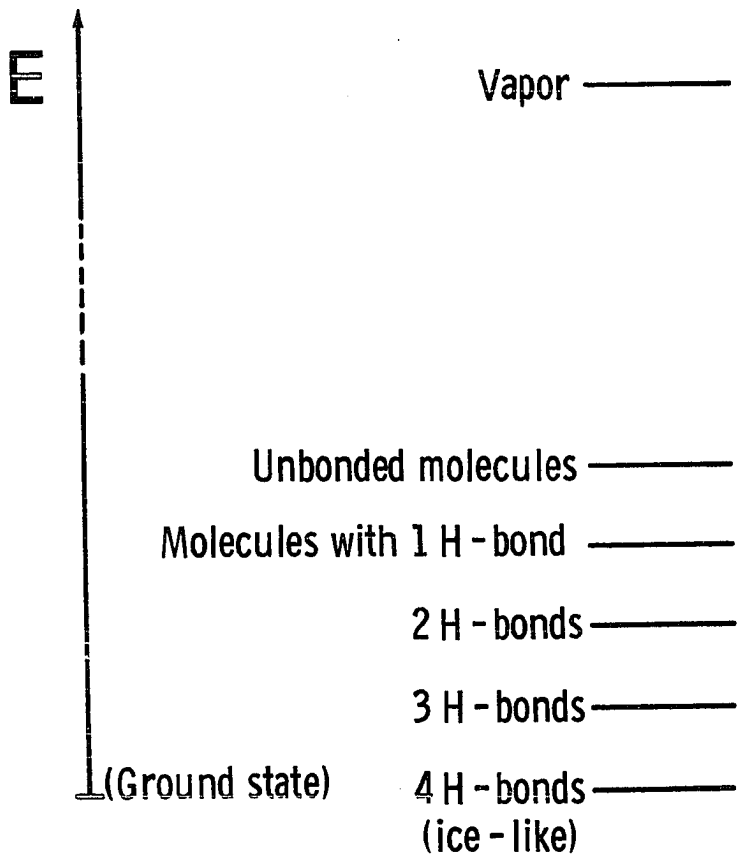

Clusters

unbonded molecules or "melt" to produce more monomeric molecules. About two-thirds of the molecules at any given instant exist as part of clusters. The molecular packing in the tetrahedrally hydrogen-bonded lattice structure of the clusters is very open. In contrast, packing in the monomeric regions is more dense, and the molecules have a higher number of nearest neighbors.

Though the model includes only two main structures, the molecules themselves can be divided into five classes of varying energy and internal freedom, depending on the number of hydrogen bonds in which they are participating (Nemethy and Scharaga, 1962a). This is illustrated in Figure 3. Molecules in the interior of the clusters are hydrogen bonded to four other molecules. On the surface of the clusters, molecules may be hydrogen bonded to one, two, or three other molecules. And of course, the molecules in the space between the clusters are not hydrogen bonded to any other molecules.

Basic to all of these "mixture" theories is the idea of an equilibrium between the various forms of water structure, an equilibrium which can be shifted by factors such as temperature or the addition of nonpolar solutes. The aqueous environment is a dynamic one. The water clusters are not permanent, rigid structures, but rather "flickering" clusters which are constantly melting and reforming. The point is that the clusters form and break down with a frequency 100

Figure 3. Schematic representation of energy levels for water molecules in liquid water. Taken from Nemethy and Scheraga (1962a).


E
Vapor
Unbonded molecules
Molecules with 1 H - bond
2 H - bonds
3 H - bonds
(Ground state)
4 H - bonds
(ice - like)

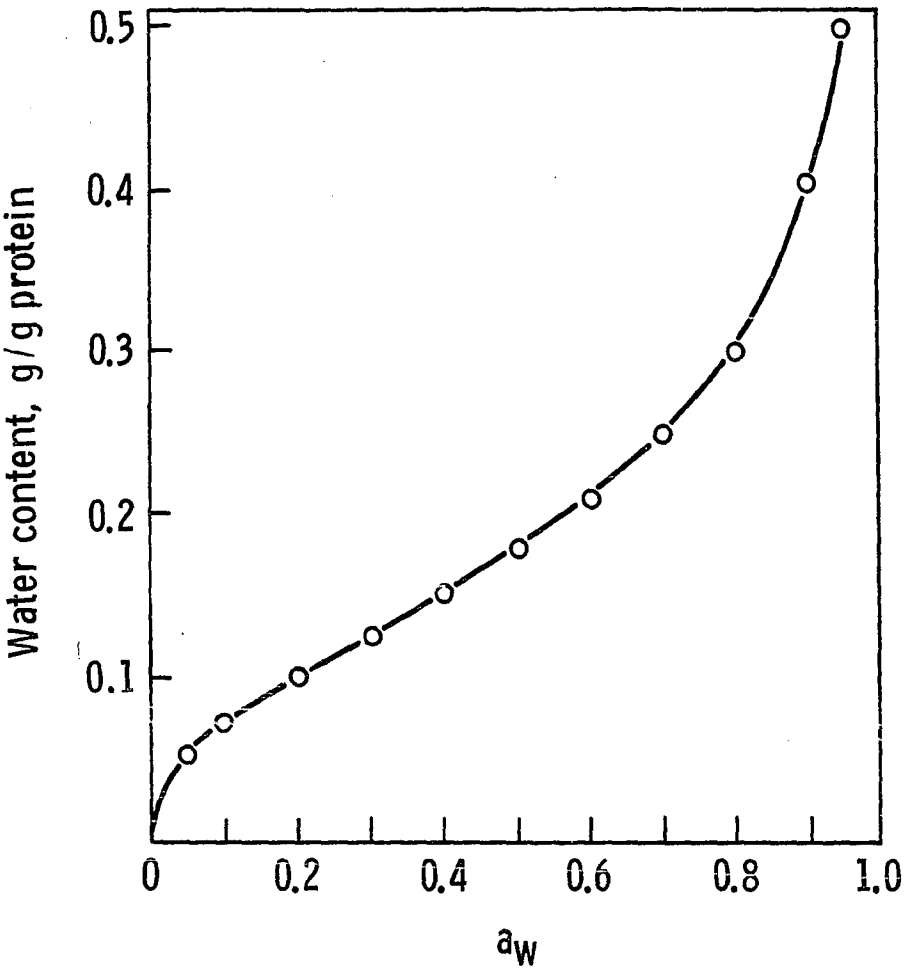
to 1000 times less than that with which molecules vibrate around their equilibrium positions within the clusters. Thus, the molecules may oscillate hundreds of thousands of times around their equilibrium positions in a cluster before the cluster falls apart. And, the constituent molecules quickly reform new clusters (Drost-Hansen, 1966).

Bound water and water activity

Probably the most common way of studying the water in food systems is to examine their isotherms. An isotherm is a graph of water activity as a function of water content at constant temperature. Water activity (a_w) is the equilibrium vapor pressure of the food divided by the vapor pressure of pure water. A typical isotherm is shown in Figure 4. An isotherm can be divided into three loosely-defined zones (Fennema, 1976). Zone I comprises the region from a_w 0-0.25; zone II the region from a_w 0.25-0.8, and zone III the region from a_w 0.8-1.0. Pure water has an a_w of 1.0.

Zone III water is the first water removed in the dehydration process. An a_w of 0.8 generally corresponds to a water content of 12-25 percent (wet basis) although this may vary greatly with the composition of the food. Most of this water is available to support growth of microorganisms and to mobilize reactants in chemical reactions. This water zone includes water physically entrapped in foods by tissue

Figure 4. A typical isotherm for a protein.


Water content, g/g protein
0.5
0.4
0.3
0.2
0.1
0
0.2
0.4
0.6
0.8
1.0
a_w

matrices, membranes, macrocapillaries (over 1μ diam), etc. The water holding capacity of a food refers to the amount of this category of water that it can hold via physical immobilization.

Zone II water comprises the next water removed in the dehydration process down to about 3-7 percent water, which corresponds to monolayer coverage of the food surface. Most "dry" food products have a water content in this 3-7 percent range. A major proportion of zone II water is unfreezable. This category includes water in microcapillaries (less than 1μ diameter), water hydrogen bonded to solutes, and water hydrogen bonded in multilayers to other water molecules adjacent to food surfaces.

Zone I water is the water directly adsorbed to polar or ionic sites on food surfaces and the water that is part of chemical hydrates. All of this water is unfreezable.

The concept of bound water is related to the concept of water activity, although the two terms are not synonymous. Water activity is a measure of the vapor pressure of the water in a food. Hence, water activity will be lowered when the binding of water to food constituents or the other water molecules is so strong that its vapor pressure is lowered.

Bound water in a food system is generally defined as water with properties detectably different from those of the "bulk" water in the same system (Kuntz and Kauzmann, 1974).

It is unlikely that there is a sharp, physical boundary separating "bound" from "bulk" water, since only relatively weak interactive forces are involved (Kuntz and Kauzmann, 1974). Perhaps it is more realistic to visualize a continuum from very tightly bound water on the food surface to bulk water in the intersticies with water bound to varying degrees in-between.

Measurement of Bound Water

Nuclear magnetic resonance

Many workers have used nuclear magnetic resonance (NMR) to study the water in protein and food systems. The use of NMR to study water is based on hydrogen atoms possessing a magnetic moment and lining up in a magnetic field. At certain magnetic field strengths and radio frequencies of irradiated energy, hydrogen atoms will absorb energy and change their orientation in the magnetic field in the process. Several different types of NMR experiments can be done. All of them provide information about the mobility and electronic environment of the hydrogen atoms in water.

Steady-state NMR In steady-state NMR spectroscopy, the sample is irradiated continuously with radio frequency energy (Cope, 1969). Steady-state experiments can be done using either high-resolution or wide-line instruments. Hydrogen atoms whose molecular motion is restricted, such as those in solids, exhibit

broad resonance lines, while those whose motion is less restricted, such as those in liquids, give narrower peaks (Dyer, 1965).

Kuntz et al. (1969) obtained high-resolution NMR spectra from frozen solutions of bovine serum albumin (BSA) hemoglobin, ovalbumin and lysozyme. The solutions contained 90-95 percent water and were frozen at -35 C. The narrow NMR signals from the samples were assumed to be coming from that water which did not freeze, and were attributed to bound water. It was noted that the linewidths of these signals were intermediate to those of ice and bulk water, showing that the water being measured was more mobile than solid ice, but clearly less mobile than liquid water at the same temperature (Kuntz et al., 1969). The water being measured was described as "constrained but with no definite long-range organization". The amounts of water bound to lysozyme, ovalbumin, and BSA were found to be 0.36, 0.31, and 0.37 g H_2O/g protein, respectively.

In later work, Kuntz (1971) obtained similar NMR spectra of polypeptide solutions containing 90-95 percent water at -25, -35, and -45 C. He found ionic and polar groups to be much more heavily hydrated than nonpolar groups. Hydration per mole was independent of polymer concentration. Based on these data and extrapolation from titration curves, Kuntz assigned a hydration value, expressed in moles of water per

mole amino acid, to each type of amino acid. He found that the hydration of proteins and polypeptides, as measured by high-resolution NMR, could be predicted from these values if their amino acid compositions were known. In other words, the water bound by proteins was the sum of water bound by the individual constituent amino acids.

Kuntz's view of protein hydration as simply the sum of individual amino acid hydrations via hydrogen bonding to polar and ionic groups may be over-simplified. Kuntz attributes water binding to hydrogen bonding to ionic and polar side chains (Kuntz, 1971; Kuntz and Zipp, 1977). This is said to explain why protein hydration can be correlated to amino acid composition. Kuntz also agrees that bound water is that water which does not freeze (Kuntz et al., 1969; Kuntz, 1971; Kuntz and Zipp, 1977). Yet Kuntz states that there is no unfreezable water in amino acid solutions (Kuntz and Zipp, 1977). If water binding were simply a matter of hydrogen bonding to polar or ionic groups, surely water would bind to amino acids and thus be unfreezable.

Hazlewood et al. (1969) studied the water in skeletal muscle containing 80 percent water at 32-37 C using high-resolution NMR. Their conclusions were based on the fact that the width of the signal produced by water hydrogens is dependent on the motional freedom of the water molecules. They concluded that essentially all skeletal muscle water

experiences restricted motional freedom compared to free water. Water in muscle tissue produced two NMR signals, one of which was much broader than the other, but both of which were significantly broader than the signal produced by free water. The broadest signal, representing about 10 percent of the total water, was attributed to water very strongly bound to the muscle protein.

Thus, 90 percent of the water in the muscle tissue experienced a degree of motional freedom between that of free water and the most strongly bound water (Hazlewood et al., 1969). It was concluded that this water is more ordered than free water. The water hydrogens in this phase exchange rapidly among themselves and with those of free water added to the system. When free water was added to a sample tube containing skeletal muscle, the resulting single water peak was narrower than that of muscle water, but still broader than that of pure water. This intermediate width is consistent with exchange narrowing. This means that if restricted water molecules or their hydrogen nuclei exchange rapidly (on an NMR time scale) with added free water molecules, the resulting NMR signal will be a single peak with a width which reflects the average environment of the hydrogen nuclei.

Hazlewood et al. (1969) emphasized that if added free water did not exchange with the restricted water, it would be visible on the NMR spectrum as a separate narrow line

superimposed on the broader muscle water peak. Neither the inhomogeneity or the viscosity of the bulk muscle phase would prevent the appearance of the sharp, narrow free water signal if free water were present. Thus, water hydrogens in the restricted phase do exchange with those of free water added to the system.

Toledo et al. (1968) used wide-line NMR to determine the amount of bound water in wheat flour systems with moisture contents varying from 10.02 to 59.5 percent. The dry flour contained 6.7 percent protein. NMR readings were taken at temperatures varying from -55C to +50 C. In this study, bound water was defined as the water equivalent to the NMR signal at -18 C. Water with very restricted mobility, such as ice, did not produce a signal. Only more mobile water, such as that remaining unfrozen at subfreezing temperatures, produced an NMR signal. The signals of samples at all moisture contents at or above 24.6 percent water at subfreezing temperatures were the same. It was therefore concluded that the amount of water bound by a given weight of dry matter was independent of moisture content. This amount was 0.32 g water/g solids (24.6 percent water).

A different wide-line technique was used by Shanbhag et al. (1970) to study water binding in wheat flour, corn starch and egg white at 22 C. This technique was based on data obtained using different attenuation (db) levels. Both free

and bound water produced signals at 28 db. Free water (as pure distilled water) did not produce a signal at 0 db. All types of systems containing 25 percent water or less (all water at these moisture levels is bound) produced the same reading, in NMR units per gram of water, at 0 db. Since the water producing a signal at 0 db in these low-moisture systems was known to be bound water, and since pure distilled water was known not to produce a signal at this attenuation level, the signal at 0 db was used to determine the bound water content of systems at all moisture levels. Each type of food was studied at moisture contents ranging from 5-85 percent water. For each system, the signal at 0 db increased up to a certain moisture content, then remained constant, regardless of how much more moisture was added. These moisture contents, henceforth called critical moisture contents, for starch, flour, and egg white, were 20.6, 24.8, and 26.1 percent, respectively. The authors stated that these critical moisture contents were bound water capacity (BWC) of these foods. It was concluded that the BWC was independent of moisture content above these levels of water.

Okamura et al. (1978) used this same technique to measure the BWC of soy flours at 21 C. The signal at 0 db for all flours reached a maximum at a critical water content, then remained constant regardless of water content. Both defatted and full-fat soy flours and flakes with moisture

contents up to 50 percent were studied.

Pulsed NMR In pulsed NMR techniques, unlike NMR where the sample is irradiated continuously, the sample is irradiated with pulses of radio frequency energy, and radio emission is measured following the incident pulses (Cope, 1969). Pulsed NMR is often used to measure NMR relaxation responses, which can provide information about bound water. Two types of relaxation responses can be measured. Both types can be visualized if one considers a collection of magnetic nuclei at equilibrium in a magnetic field, with their nuclear spins partially aligned by the field, and the sample displaying a macroscopic magnetic moment along the axis of the magnetic field (Kuntz and Kauzmann, 1974). If this equilibrium is disturbed by a radiofrequency field, the magnetization of the sample will relax exponentially to a new value with a characteristic time constant, T_1. This type of relaxation process, called spin-lattice relaxation time, involves energy transfer between the sample and the environment. The second relaxation process involves relaxation of magnetization perpendicular to the direction of the magnetic fields. Since there are no preferred alignments perpendicular to the field, there is no energy exchange with the environment. Instead, the relaxation involves spin-spin interactions and is called spin-spin relaxation time, or T_2. This relaxation time is proportional to the molecular mobility of water molecules,

Cope (1969) used pulsed NMR to study the T_2 values of the water in rat muscle. He found, as Hazlewood et al. (1969) had with steady-state NMR, that the tissue water consisted of two distinct fractions with markedly different values of T_2, both of which were much shorter than the T_2 of free liquid water. Essentially none of the tissue water possessed values of T_2 like those of bulk water. The fraction with the shortest T_2, the most tightly bound fraction, comprised 27 percent of the total water. The likelihood that each of these two fractions of tissue water is not homogeneous, but may consist of two or more subfractions with different structures was expressed.

Hansen (1976) used two pulsed NMR methods to study the state of water in soy protein concentrate (SPC) and ovalbumin. First T_2, the spin-spin relaxation time, of water in hydrated protein samples was measured. Rapid exchange between free and bound water was assumed. At water contents below 0.07 g/g solids (6.5 percent water), T_2 became too short to be accurately measured because this water is so tightly bound. Relaxation times for water in SPC samples with water contents up to 0.26 g water/ g solids (20.6 percent water) were very short and nearly constant, increasing only slightly with increasing water content. Above this water content, T_2 began increasing rapidly with increasing water content. In ovalbumin systems, the low T_2 values prevailed at water contents

up to 0.20 g/g solids (16.7 percent water), at which point T_2 values began increasing rapidly with increasing water content. Thus, this change in T_2 occurred at a slightly lower water content in ovalbumin systems than in SPC systems. From these data, it was concluded that water up to 0.26 and 0.20 g/g solids for SPC and ovalbumin, respectively, is bound water and that any additional water is more like free water. T_2 values for free water were not measured or discussed.

Hansen (1976) noted that the bound water contents determined by this NMR method were in agreement with those determined from the Bradley isotherm treatment of adsorption isotherm data for SPC and ovalbumin, suggesting that these different techniques were identifying the same water species. The Bradley isotherm treatment predicted the bound water content of SPC to be 0.26 g/g solids (20.6 percent water).

Bradley's sorption equation is based on the assumption that the first layer of molecules is sorbed with a net orientation of dipole moments induced by the polar surface (Bradley, 1936). This adsorbed (polarized) layer in turn polarized another layer, which polarized a third, and so on. In this way a polarized multilayer of molecules is built upon the surface. The Bradley equation in linear form is: $\log (\log 1/a) = \log K_2 + V \log K_1$. K_1 and K_2 are constants which are functions of the dipole moment of the sorbed vapor and of the sorptive polar groups, respectively. V is the amount of vapor sorbed and "a" is the activity of the sorbed vapor.

Bradley plots of log (log 1/a) vs. V for SPC adsorption data were linear up to a water content of 0.25 g/g solids (20 percent water), Hansen (1976). Above this water content (corresponding to an a_w of 0.85), the experimental points diverged from linearity. This behavior is consistent with multilayer sorption of water in SPC systems up to 0.25 g/g solids (Hansen, 1976; Bradley, 1936).

Hansen (1976) also measured the water NMR signal amplitudes in SPC and ovalbumin systems at temperatures above and below 0 C. No signal was produced by water with very restricted mobility. It was assumed that all of the water which did not produce a signal at -50 C was in the form of ice. The water which was mobile enough to produce a signal at -50C was assumed to comprise all of the bound (unfreezable) water in the systems. Ovalbumin and SPC systems containing 1 and 2 g water/g solids (50 and 67 percent water) were studied. The signal amplitude at -50 C was the same at both moisture levels in both systems. The data indicated that the bound water contents of SPC and ovalbumin were 0.26 and 0.33 g/g solids, respectively, and were independent of water content. This bound water content was the same as had been determined from the T_2 values and the Bradley isotherm treatment of the adsorption data.

In a later study, Hansen (1978) also measured NMR signal amplitudes at temperatures above and below 0 C, and noted that

it was essentially the same method that Kuntz (1971) had used. The amplitudes of the signals produced at -50 C by hydrated ovalbumin, SPC, and soy protein isolate (SPI) were measured. The water binding capacities of ovalbumin and SPC were found to be independent of water content between 0.3 and 2.0 g total water/ g solids (23 to 67 percent water). The bound water contents of ovalbumin and SPC were 0.26 and 0.33 g/g solids, respectively. The water binding capacity of SPI more than doubled as total water content was increased from 0.3 to 3.0 g/g solids (23 to 75 percent water). The bound water contents of one isolate were 0.35, 0.46, and 0.47 g/g solids at water contents of 49, 66, and 70 percent, respectively. At the higher water contents (up to 75 percent), the water binding of SPI leveled off and appeared to approach a maximum value of about 0.49 g/g solids. Hansen (1978) stated that this maximum value was the same value calculated from the water-binding abilities of the individual amino acids according to Kuntz's (1971) method. He then concluded that this value must represent full hydration of the water-binding sites.

The fact that the bound water content of SPI increased with increasing water content up to 75 percent water, while that of SPC and ovalbumin did not increase, was attributed to swelling of the isolate with increased hydration tc expose more water binding sites (Hansen, 1978). The fact that the

soybean cellular structure is still intact in SPC may prevent its swelling to expose more water binding sites.

Calorimetry

Differential scanning calorimetry (DSC) has been defined by the International Confederation for Thermal Analysis (ICTA) Nomenclature Committee as "a technique for recording the energy necessary to establish a zero temperature difference between a substance and a reference material against either time or temperature, as the two specimens are subjected to identical temperature regimes in an environment heated or cooled at a controlled rate" (MacKenzie, 1969). The sample and reference pans are equipped with separate heating elements and have separate temperature sensing devices. The two pans are maintained at the same temperature through electronic control of the rate at which heat is electrically supplied to them (Pope and Judd, 1977). Thus, DSC is different from conventional differential thermal analysis (DTA), in which a sample and inert reference material are subjected to the same controlled heating program and the difference in their respective temperatures is measured (Watson, et al. 1964). Many of the applications of DTA and DSC are similar, although only DSC can be used for isothermal work. Both are used to determine heats and temperatures of transitions (Daniels, 1973). The major distinction between DTA and DSC as well as

the major advantage of DSC is that the amplitude of the pen from the baseline position is directly measurable as a rate of energy output or input (millicalories per second) and the area under a peak equals the total transition energy in calories (Watson, et al. 1964). Measurement of transition energy is not affected by either sample geometry or sample heat capacity. DTA peaks do not provide a direct measurement of heats of transition, although their areas are proportional to heats of transition.

Another disadvantage of DTA (but not of DSC) is that the thermal conductivity of the DTA sample markedly influences the peak area obtained per unit of energy input or output (Watson, et al. 1964). This problem is handled in practice by diluting the sample with a large volume of the reference material so that the resultant conductivity is essentially equal to that of the pure reference (Barrall and Rogers, 1962). This dilution can decrease the sensitivity of the method. In contrast, no actual reference material is required for DSC. Only an empty sample pan is placed in the reference well.

Calorimetric measurement of bound water.

Dehydration Karmas and DiMarco (1970) used DSC to isothermally dehydrate protein dispersions containing 73-75 percent water at 105 C. They used open sample pans. The

details of their procedure were omitted. They did not explain how it was possible to use open pans containing 2 mg of sample that were placed on a sample holder already heated to 105 C and yet to get accurate recordings of the energy used to vaporize the water. Most of the water in open sample pans would evaporate instantly at 105 C.

Karmas and DiMarco (1970) calculated the ratio of the average energy required to evaporate 1 mg water from each protein dispersion to the energy required to evaporate 1 mg distilled water. This ratio was called the water binding index (WBI). A higher ratio was said to indicate a greater degree of water binding by a protein. Soy isolate at pH 7.0 had a WBI of 1.33, while soy isolate at pH 4.6 had a WBI of 1.09.

This WBI value could possible be used to indicate the relative water binding abilities of different substances as long as the water contents of the samples were the same. However, the WBI values would be difficult to interpret if one were comparing samples with different water contents. Samples with higher water contents, because of their greater absolute amounts of free water, would have lower WBI values. But this would not necessarily mean that they contained less bound water.

Another problem with this isothermal dehydration method, which Karmas and DiMarco (1970) themselves mentioned, is that tightly bound water is not removed during this very short time

of exposure to a temperature of 105 C.

Karmas and Chen (1974) used the same isothermal dehydration method as had been used by Karmas and DiMarco (1970) to determine the percent of bound water present in soy protein isolate and sodium caseinate dispersions. The water contents of the dispersions ranged from 75-95 percent. The resultant endothermic curves consisted initially of sharp, tall peaks that then tailed out and returned to the baseline gradually. The initial sharp peaks were arbitrarily extrapolated to the baseline to enclose an area which included only the sharp peak and not the rest of the curve area. This sharp peak area was measured and assumed to have resulted from the vaporization of free water only. The other portion of the curve was assumed to have resulted from the vaporization of bound water and was not measured. No evidence to justify this seemingly arbitrary division between bound and free water was presented. Hence the results must be viewed with skepticism.

The area of the sharp-peak part of each curve (Karmas and Chen, 1974) was used to determine the weight of free water in the sample. This weight was subtracted from the total water weight to determine the amount of bound water present. The weight of bound water was expressed as a percent of total water decreased, since there was less free water present in the samples at the lower water contents.

Occurrence of fusion Both DTA and DSC have been used

to determine the amount of unfreezable water in food systems. The definition of bound water as being that portion of the water in a food which does not freeze is widely accepted.

Duckworth (1971) used DTA to measure the thermal transitions in several food products from -78 C to above 0 C. Moisture contents of the products ranged from about 0.22 to 0.35 g/g solids (18 to 26 percent water). For every food, there was a characteristic moisture level below which no endothermic peaks attributable to melting of ice were detected. Since all of the water at these moisture levels would be bound and therefore unfreezable, this is not surprising. At moisture contents above this characteristic level, all foods showed endothermic peaks attributable to melting of ice, indicating the presence of some free water. The size of these peaks increased as total moisture content increased. This critical moisture content varied from one food to another. For peas, cod muscle, and potato, the critical levels were 0.195, 0.265, and 0.23 g water/ g solids, respectively. These correspond to moisture contents of 16.3, 21, and 18 percent.

These moisture levels were assumed to be the maximum amount of water that these foods would bind. As soon as this critical water content was attained, all additional water was assumed to be free water. Duckworth stated, without apparent supportive data, that the results "unquestionably confirm

that, for each of the materials examined, there exists a definite and fixed amount of water of hydration which is incapable of undergoing a normal freezing process. Additional water above this level freezes in a normal manner."

Parducci and Duckworth (1972) reported a similar study on egg white, cod, and celery with similar conclusions.

Ruegg et al. (1974) used DSC to determine the amount of unfreezable water in hydrated casein systems. Casein samples with water contents ranging from 0.3 to 5.0 g/g solids (23 to 83 percent water) were cooled to -40 C and then heated to 10 C. Using the curves that resulted from those scans, two methods were used to determine the bound water content of the systems. The first method was the same as the Duckworth method already described. The highest water content which produced no endothermic peak due to melting of ice (zero heat of fusion) was considered to represent the maximum water binding capacity. These values were 0.429, 0.442, and 0.430 g/g solids for micellar, rennin treated, and acid precipitated casein, respectively.

Measurement of freezable water The second method that Ruegg et al. (1974) used to measure bound water was based on the assumption that the heat of fusion of the freezable water was equal to the heat of fusion of pure water, 79.7 cal/g. The peak areas were measured to determine the millicalories used to melt the frozen water. This value was divided by

79.6 to determine the amount of free water present. The total water contents were determined by puncturing the lids of the sample pans following the scans and drying over phosphorus pentoxide in vacuo. The amount of bound water was calculated as the difference between the total and freezable water contents. It was stated that the results of this method and the first method were the same, but the data for these systems at the various water contents were not presented.

Berlin et al. (1970) used DSC to measure the unfreezable water in hydrated casein, BSA, and B-lactoglobulin. The samples were frozen at -40 C and then heated to 20 C. Bound water content was determined by subtracting the freezable water from the total water present. The amount of freezable water was calculated using the value of 79.6 cal/g for its heat of fusion. The total water content in each sample pan was determined following the scan by puncturing the cover of the pan and drying the sample under vacuum. Reported water contents of the casein systems varied from 69 to 73 percent. The average bound water content of the casein systems was 0.553 g/g protein and did not vary significantly in this moisture range. Water contents of the BSA systems varied from 48 to 52 percent. Their average bound water content was 0.488 g/g protein. The water content of the β-lactoglobulin systems was 65-66 percent. Their average bound water content

was 0.553 g/g protein.

Berlin et al. (1973) used this same method to determine the bound water content of whey protein concentrates containing 65-75 percent water. The exact water contents of individual samples were not given. These whey protein systems contained 0.45-0.52 g bound water/g protein.

Bushuk and Mehrotra (1977) used DTA to determine the bound water content of wheat flour doughs with moisture contents up to 50 percent (1.0 g/g solids). The samples were frozen at -30 C, and the bound water content was calculated by subtracting the amount of freezable water from the total water.

Freezable water was graphed as a function of total water. A roughly linear plot with a slope of 0.65, and which intersected the x-axis at 0.3 g water/g solids (24.7 percent water), was obtained. The graph showed that all water up to 0.3 g/g solids was bound. If all additional water above this level had been free, the slope of the graph would have been 1.0. The slope of 0.65 indicates that about two-thirds of the added water above this level was free, and one-third was bound.

A graph of the bound water content of these same samples as a function of total water showed a relationship between the two parameters which appeared slightly curvilinear, rather than strictly linear, as the authors had thought at first. The slope of the graph appeared to increase with increasing

moisture content, particularly above 0.6 g/ g solids (37.5 percent water). This means that an even larger proportion of the added water may have become bound water at the higher moisture levels (between 0.6 and 1.0 g/g solids). The authors were unable to explain the increase in bound water with increasing moisture content, as this finding conflicted with conclusions of other workers. They suggested that perhaps additional moisture uncovered new sites for water binding that were not accessible initially, but concluded that further research was necessary to clarify this point.

Biswas et al. (1975) used DSC to measure the bound water content of aqueous solutions of various sugars and complex carbohydrates. Samples were cooled to -73 C, then heated to 20 C. Bound (unfreezable) water was determined by subtraction, as has been described. The amount of bound water associated with each type of carbohydrate increased as the water content of its solution increased. Sucrose, lactose, and maltose solutions contained about 0.75 g bound water/ g solids at water contents of 60-70 percent. At higher water contents, the bound water increased rapidly, reaching 3.0 g/g solids at 90 percent water, and 9.0 g/g solids at 95 percent water. Glucose solutions up to 70 percent water contained 0.4 g bound water/g solids. At higher water contents, the bound water increased rapidly, reaching 4.0 g/g solids at 99 percent water. It is noteworthy that the most rapid increases

occurred at moisture contents above 70 percent. Complex carbohydrates such as carboxymethylcellulose, guar gum, and locust bean gum, exhibited huge increases in unfreezable water with increasing dilution at water contents above 99 percent. One type of CMC bound 160 g water/ g solids is a solution containing 99.9 percent water.

Biswas et al. (1975) concluded that the amount of water bound to the solute was dependent on the water activity of the solutions. They postulated that an equilibrium exists in the solutions between bound and free water and that additional water shifts the equilibrium so that more water becomes bound to the solutes.

Ross (1978) used DTA to measure the unfreezable water in three types of systems. Samples were cooled to -70 C and the bound water content was calculated by subtracting free water from total water. The first system contained corn oil, carboxymethylcellulose, Na-caseinate and glycerol in a weight ratio of 1:12:12:25. Samples had moisture contents ranging from 28.6-80.0 percent. Over this entire range, the amount of water bound per gram solids increased with increasing water content, reaching about 1.2 g/g solids at 80 percent water (4.0 g/g solids).

The second system contained ground beef, soy flour, sucrose, NaCl, and lard (11:13:18:1.5:1) at moisture levels from 29.5 to 47.5 percent. From 29.5 to 33 percent water, the bound water content remained constant at 0.40 g/g solids.

Above 33 percent water the bound water content increased rapidly with increasing total moisture, reaching 0.6 g/g solids at 47.5 percent water (0.9 g/g solids).

Sodium-caseinate dispersions at moisture contents from 50 to 98 percent also were studied. Bound water increased consistently with increasing total water, from about 0.55 to about 1.35 g/g solids.

Ross (1978) concluded that the amount of bound water per gram of macromolecule increases concomitantly with increasing a_w, and that water binding is an equilibrium process. Assuming it is an equilibrium process, then the law of mass action dictates that an increase in the activity of one reactant (free water) causes an increase in the activity (and thus concentration) of the product species (hydrated solute).

Dielectric measurements

The polarity of liquid water molecules influences the ionization of ionic bonds or highly polar covalent bonds (Neal, 1971). For example, the greater the polarity of water or some other solvent, the greater is its ability to neutralize the attraction between the atoms of a polar covalent bond and cause dissociation. This property of a solvent which keeps ions separated is measured and expressed as the dielectric constant of the solvent. It is a measure of relative polarity.

The dielectric behavior of aqueous protein solutions has been extensively studied over a frequency region extending from a few kilohertz to tens of gigahertz (South and Grant, 1972). The dispersion occurring at the lowest frequency (0.1 to 10 MHz), the β dispersion, is attributed to the protein. The highest frequency dispersion, the γ dispersion, is attributed to free water. A bound water dispersion, the δ dispersion, occurs at intermediate frequencies (Grant, 1966). Bound water exhibits dielectric relaxation at intermediate frequencies ranging from a few to several hundred MHz. The dielectric constant of bound water is 5, so is much lower than that of free water, which is about 80 (Grant, 1966; Grant et al., 1974).

Grant et al. (1974) used dielectric methods to measure the hydration of myoglobin in solutions of different concentrations. The parameters used to calculate the degree of hydration were the permittivities (dielectric constants) of the high and low-frequency limits of the δ (bound water) dispersion. All of the solutions studied were quite dilute, containing 92.3, 90.1, and 83.9 percent water. The data were used to calculate the amount of bound water using two methods. Both methods showed a slightly greater degree of hydration at the lower protein concentrations (higher water concentrations). The authors concluded that it was possible that the proximity of protein molecules affected their interaction

with the solvent and that clearly, since there is a finite amount of water in the system, the degree of hydration of each protein molecule must surely fall at some state as the water content is decreased. They concluded that further study of this possibility was necessary.

Vaporization of Water

Heat of vaporization

Berlin et al. (1970) also used DSC to measure the integral heat of vaporization of the water in four proteins hydrated to varying degrees. In all four protein systems, the heats of vaporization (ΔH_v) at water contents above 0.18 g/g protein were higher than at 0.18 g/g. BSA data also showed that the ΔH_v at a very low moisture level (0.055g/g) was higher than at 0.18 g/g.

When the water content of casein was increased from 0.178 to 0.187 g/g protein (15 to 15.7) percent), the ΔH_v increased from 571 to 678 cal/g. An increase in the water content of collogen from 0.134 to 0.240 g/g (11.8 to 19.4 percent) resulted in an increase in ΔH_v from 594 to 675 cal/g. The ΔH_v reached 718 cal/g at 0.348 g water/ g protein. The ΔH_v of B-lactoglobulin increased from 530 to 677 cal/g with a water content increase of 0.157 to 0.213 g/g (13.6 to 17.6 percent). The ΔH_v of water sorbed to BSA was 667 cal/g at a water content of 0.055 g/g protein, and decreased to 576 cal/g at 0.162 g/g. The ΔH_v increased to 651 cal/g at 0.214 g

water/ g protein, and increased further to 671 cal/g at 0.453 g water/g protein.

Berlin et al. (1970) stated that the observed increase in ΔH_v after about 0.18 g water were sorbed per gram protein was unexpected, since a decreasing heat of adsorption generally is observed with increasing surface coverage. In fact, the data at low moisture levels do show this decrease in ΔH_v. At low hydration levels, water is bound directly to specific hydrophilic sites. As more water is bound in multilayers and the original monolayer, the binding is weaker and the ΔH_v decreases.

It was suggested that once a critical amount of water is sorbed, the sorbed water may form a quasi-solid, "icelike" structure, Berlin et al. (1970). This critical moisture level for the proteins in this study was above 0.18 g/g. The water in this quasi-solid structure would have a heat of vaporization higher than that of water that was simply indirectly hydrogen bonded in multilayers to protein hydrophilic sites.

Berlin et al. (1970) pointed out that while entropy changes (ΔS) could not be calculated from the DSC data, the formation of a quasi-solid water structure should yield negative ΔS values because of the increased order of the system. This water structuring may be comparable to that which occurs when a nonpolar solute is introduced, a phenomenon which is accompanied by a decrease in entropy. Berlin et al. (1970)

suggested that these ideas fit in well with the hydrogen bonded cluster theories of liquid water.

Berlin et al. (1971) measured the heat of vaporization of water from whey, skimmilk, and whole milk powders. The data confirmed the results of their earlier study. When the protein powders sorbed a sufficient amount of water, the heat of vaporization of that water increased by 80 to 136 cal/gram.

Vaporization curves

Karmas (1968) used a DSC 1B to dehydrate amino acid solutions containing at least 95 percent water. The temperature was increased at a rate of 10 degrees C per minute up to 140 C. The temperature at which the program was started was not given. Karmas placed the samples in open sample pans, rather than sealed sample pans. Since the sample size was 3 µl, sample evaporation could have occurred prior to the beginning of the scan. Hence, there may have been some error in the total water weight used in the calculation.

All of the amino acids produced large endothermic peaks at about 70 C. Six amino acids--arginineHCl, aspartic, cystine, glutamic, glutamine, and tyrosine--produced no additional peaks at higher temperatures. The additional (secondary) peaks of four amino acids, isoleucine, leucine, methionine, and valine, were especially large; their areas constituted 30 to 70 percent of the total endothermic peak areas of these

amino acids. In contrast, the areas of the secondary peaks of asparagine, cysteine, histidineHCl, lysineHCl, proline, serine and threonine constituted only 1-6 percent of their total endothermic peak areas.

The large secondary peaks produced by the isoleucine, leucine, methionine and valine solutions were attributed (Karmas, 1968) to some type of water binding stronger than multilayer adsorption on polar sites. Karmas assumed that such multilayer adsorption occurred in the solutions of the polar and ionic amino acids. But, since higher temperatures were required to remove the secondary peak water from these nonpolar solutions than were required to remove water from more polar amino acid solutions, the type of binding involved was assumed to be stronger. Karmas (1968) attributed this stronger binding to the existence of a structured form of water around the nonpolar residues. Although the nature of this water structure could not be determined, Karmas (1968) suggested the possibility of quasi-crystalline clathrate cates of water being built around the nonpolar residues, as had been proposed by Frank and Quist (1961).

Karmas (1968) also used a DSC 1B to dehydrate beef muscle tissue and egg albumin. Only one endothermic peak was obtained, but the peak extended well beyond 150 C, instead of ending at 70-80 C, as had the first peak from the amino acid solutions. The high temperature required for complete

dehydration was interpreted as indicating strong water retention by these proteins.

Heat Capacity

Berlin and Kliman (1974) used DSC to measure the heat capacity of cheese whey as a function of water content. The samples were prepared by dehydrating fluid whey to varying degrees to produce systems with 7 to 93 percent water. The heat capacities of the whey systems (cal/g/^{o}C) were plotted as a function of water content. Because of the increasing amount of water present, the heat capacity naturally increased with increasing moisture content. The graph contained two linear portions, with an inflection point at 50 percent water. Below 50 percent water, the rate of increase of heat capacity with increasing water content was faster than it was above 50 percent water.

Analysis of these data yielded equations that were used to calculate apparent partial heat capacity values for the water represented by the two linear sections of the graph. The water in dehydrated whey with less than 50 percent moisture had a heat capacity of 1.203 cal/g/^{o}C, appreciably higher than that of bulk water. The partial heat capacity of the additional water in the higher-moisture systems was 0.9666 cal/g/^{o}C. It was concluded that the sorbed water exists in some structured form involving multiple hydrogen bonding.

The excess heat capacity of liquid water over that of ice or water vapor is essentially "structural" heat capacity, and is attributed to the thermal breakdown of the associated structure present in the liquid (Berendsen, 1967). Water molecules bound to isolated specific sites on the surface of a protein should not exhibit such a structural contribution to heat capacity. Therefore, the elevation of the partial heat capacity of the water up to the 50 percent level is evidence of the association of sorbed water to form a structured hydration shell (Berlin and Kliman, 1974). The fact that the partial heat capacity of the water in higher-moisture systems approaches that of bulk water is likely the result of dilution, since so much bulk water is present in addition to the structured water (Berlin and Kliman, 1974).

Berlin et al. (1972) measured the heat capacity of the water in hydrated ovalbumin systems containing 0.03 to 0.20 g water/ g protein (3-18 percent). The heat capacity of the ovalbumin-sorbed water was 1.269 cal/g/^{o}C. For the same reasons already discussed, the existence of a structured hydration shell was postulated, since the specific heat was appreciably higher than that of bulk water.

Bull and Breese (1968) used a calorimeter to measure the specific heat of egg albumin systems containing 0-100 percent water. When heat capacity was plotted as a function of moisture content, a graph with two linear portions was

obtained, similar to that reported by Berlin and Kliman (1974). The inflection point occurred at 43 percent water (0.3 g/g protein). The heat capacity of the water sorbed below 43 percent was 1.247 cal/g/^{o}C. No conclusions about structured water were drawn.

Structured Water of Hydration

NMR evidence

Fuller and Brey (1968) used NMR to study the water sorbed on BSA. The line widths of signals produced by the water in systems containing 2.4 to 24.2 percent water were measured. In this particular type of experiment, the line widths are inversely proportional to the spin-spin relaxation time for the system of nuclei. If there is rapid, random molecular motion, as in most liquids, the relaxation time is long and the line narrow. If the magnetic nuclei are fixed in their relative positions, as in a solid, the relaxation process occurs faster, and the resonance line is broader.

The NMR line widths were graphed as a function of the water content of the protein samples (Fuller and Brey, 1968). As was expected, the NMR absorption line became increasingly narrow with increasing water content. The rate of narrowing was interesting, however. Up to 0.09 g water/ g protein, the linewidth decrease was rapid. At this point the rate

of linewidth narrowing decreased drastically with increasing water content. The linewidth did continue to narrow, but at a much slower, but constant rate. At a water level between 0.15 and 0.18 g/g the width decreased again, suddenly but slightly. Above 0.18 g/g the linewidth continued to decrease but at a very slow rate.

Fuller and Brey (1968) attributed the abrupt change in the rate of decrease at 0.09 g/g to the onset, at sufficient water coverage, of a cooperative ordering of the sorbed water into some sort of structure. Such a structure would necessarily involve the formation of much more than one hydrogen bond per molecule. The usual increased freedom of motion of the protons, relative to one another, associated with increasing amounts of sorbed water would thus be offset, and the accompanying rapid decrease in line width would be slowed. The nature of the "secondary" water structure would be determined by the particular protein, but would require a different arrangement of the water molecules from that in the "primary" (first molecules bound to polar groups) water when the primary water is present alone.

The slight but sudden linewidth narrowing between 0.15 and 0.18 g water/ g protein was thought to perhaps mark the maximum amount of secondary water that could be bound by the protein (Fuller and Brey, 1968). Since the linewidth after that point continued to decrease at an even slower rate than

between 0.09 and 0.15 g/g, it seems more likely that this marked the point where some, but not all, of the additional water was unbound, free water. Up to that point all of it had been bound, and none of it free.

Berendsen (1962) studied the proton resonance signals of collagen at a_w's ranging from 0.1 to above 0.9. At a_w 0.1 narrow absorption curves were obtained. At a_w's between 0.3 and 0.8 (0.2 to 0.3 g water/ g collagen), broader curves and line splitting were observed. Above a_w 0.8-0.9 the absorption curves again became narrow.

Berendsen (1962) concluded that at a_w 0.1 each water molecule was individually adsorbed, apparently retaining a high degree of rotational freedom. At a_w's from 0.3-0.8 (0.2-0.3 g water/ g protein), the data clearly indicate that a large proportion of the molecules reorient restrictively in such a way that the resulting proton interaction occurs in or close to the fiber direction. The data suggest that the water molecules form chains, with tetrahedral angles between the bonds, in the fiber direction. At higher a_w's it was suggested that enough water is present to form structures in three dimensions, which may bear extensive structural relationships with the macromolecules. It was stated that if such structures exist, they must have many lattice defects, as indicated by the relatively narrow NMR signal, and be a state between the solid and liquid states.

Dielectric evidence

Grant et al. (1968) studied the dielectric behavior of aqueous solutions of BSA from radiowave to microwave frequencies. They found a dispersion due to bound water in the frequency range 200-2000 MHz. When the variation of dielectric decrement was graphed as a function of solute concentration, a change of slope consistently occurred between 20 and 23 percent water. Dielectric decrement refers to the difference between the dielectric constant of the solution and that of pure water. Another parameter, the difference between the dielectric constants at the low and high frequency ends of the dispersion, doubled at this same water content. These data were interpreted as indicating a sudden increase in the amount of bound water at this concentration (20-23 percent water).

MATERIALS AND METHODS

Materials

Calorimetric standards

The calorimetric standards used for temperature and power calibration were naphthalene, tin, anthracene, and distilled water. "Scintanalyzed" naphthalene was obtained from Fisher Scientific Company, Fair Lawn, New Jersey. A standard sample of tin was obtained from the National Bureau of Standards. Scintillation grade anthracene was obtained from Eastman Kodak Company, Rochester, New York.

Soy protein products

Three soy protein isolates were obtained from Ralston Purina Company, St. Louis, Missouri. Supro 610 is a hydrolyzed, high molecular weight powder that contains 95 percent protein (dry basis) and has a pH of 6.7 ± 0.3. Edipro N is a nonhydrolyzed isolate that contains 92.5 percent protein (dry basis) and has a pH of 6.7 ± 0.1. Edipro A is a nonhydrolyzed isolate that contains 92.5 percent protein (dry basis) and has a pH of 4.6 ± 0.2.

Promosoy 100, a finely ground soy protein concentrate, was obtained from Central Soya, Fort Wayne, Indiana. It contains 70 percent protein (dry basis) and has a pH of 7.0 ± 0.1.

Defatted soy flour (toasted Nutrisoy Flour-40) was obtained from Archer Daniels Midland, Decatur, Illinois.

Ovalbumin

Ovalbumin (salt-free, 99% pure) was obtained from Sigma Chemical Company, St. Louis, Missouri.

Methods

Calorimetric measurements

Calibration The Differential Scanning Calorimeter 1B was calibrated according to the manufacturer's instructions (Perkin-Elmer Corp., 1968) using haphthalene, anthracene, tin, and distilled water as standards. The range was calibrated so that the number of millicalories represented by each square inch of peak area was known. When this calibration factor was used to determine the heat of fusion of pure water, the result agreed with the accepted value, which is 79.6 cal/g. The range calibration was checked regularly, but it never changed.

The differential temperature was adjusted so that the two sample pans were at the same temperature. This calibration was checked daily, but it never changed. The average temperature was adjusted so that the temperature recorded on the chart was accurate. This calibration was checked daily, but seldom changed. A slight adjustment was necessary every month or two.

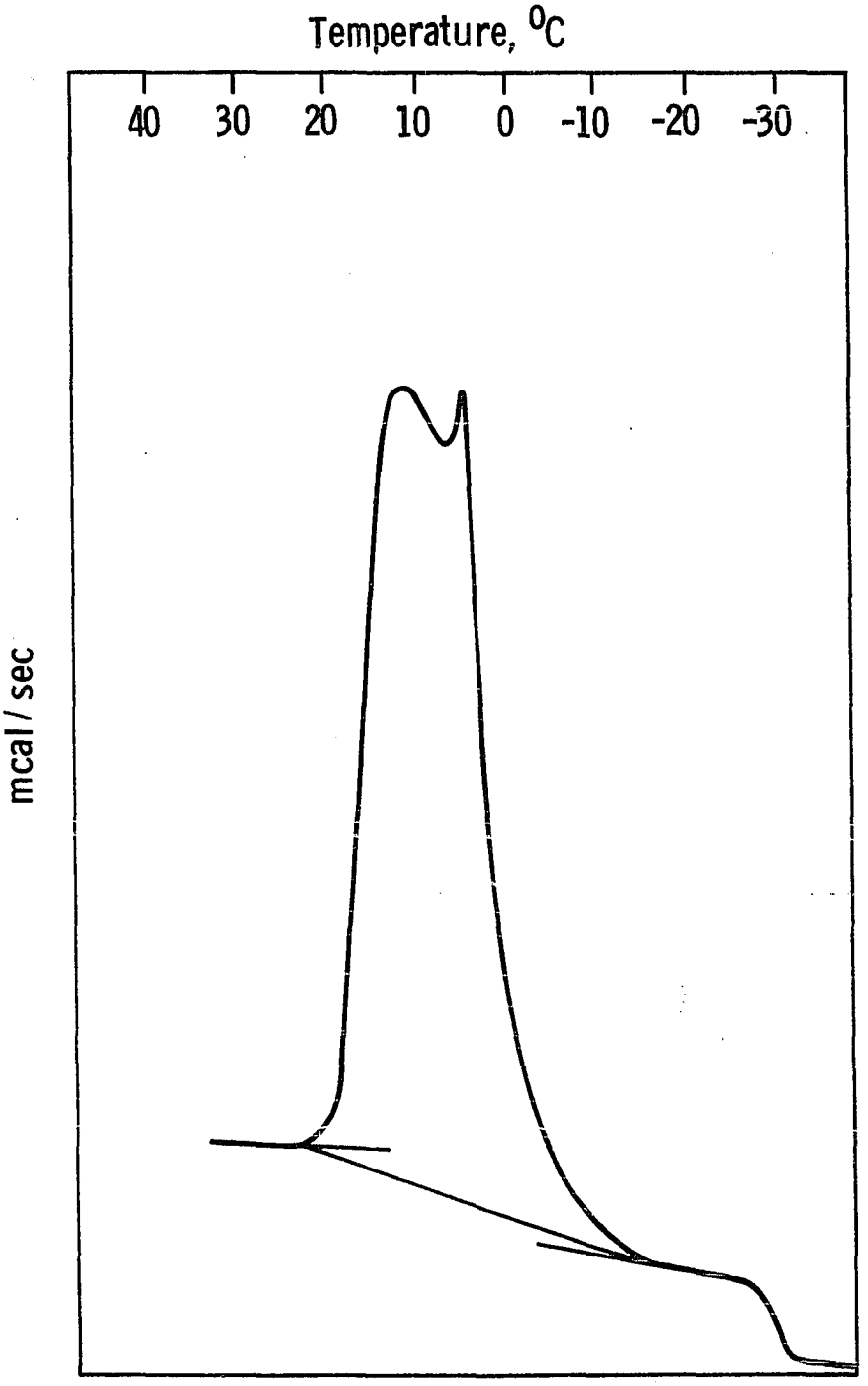
Bound water Each sample was sealed in a Perkin-Elmer volatile sample pan, weighed, and placed in the right-hand sample holder. An empty volatile sample pan was placed in the left-hand (reference) sample holder. Dry nitrogen was used as a purge to prevent condensation of moisture in the sample environment.

The low temperature cover was placed over the sample holder assembly and filled with dry ice and acetone to cool the sample to -30 C. The sample was heated at a rate of 20 degrees C per minute to at least 20 C.

Those samples that contained freezable water produced an endothermic peak upon heating because of the heat required to melt the ice. A typical peak, with the baseline drawn, is shown in Figure 5. The area of this peak was measured with a planimeter to determine the total heat of fusion (in millicalories). This value was divided by the heat of fusion of pure water, 79.6 cal/g, to determine the weight of freezable (free) water present in the sample. This weight was subtracted from the total water weight to determine the amount of unfreezable (bound) water present.

Heat of vaporization Each sample was sealed in a volatile sample pan and weighed. After the sample holder had been cooled to 17 C, the lid of the sample pan was punctured and the sample was placed in the sample holder. The low temperature cover containing dry ice and acetone

Figure 5. Example of an endothermic peak obtained by heating a frozen sample containing free water. The method of establishing the baseline is shown.


Temperature, °C
40
30
20
10
0
-10
-20
-30
mcal / sec

was then used to cool the sample to 0 C.

The sample was then heated at a rate of 20 degrees C per minute to 200 C. All samples produced an endothermic peak because of the heat required to vaporize the water present. A typical peak, with the baseline drawn, is shown in Figure 6. The area of this peak was measured with a polar planimeter to determine the total energy (in millicalories) used to vaporize the water from the samples. This value was divided by the total weight of water evaporated to determine the integral heat of vaporization in cal/g.

Each sample was weighed immediately after the scan. The difference between the weights before and after the scan was the total water weight, since further drying at 105 C produced no weight change. Scanning to 200 C caused all of the water to vaporize.

Water content

To determine the total water content of a sample for calculation of bound water content, the lid of the sample pan was punctured. Then the sample was dried to constant weight in a convection oven at 105 C.

Sample preparation

Samples that contained less than 40 percent water were prepared by humidification over distilled water in a vacuum desiccator at room temperature. Water was added directly to produce samples with higher moisture contents.

Figure 6. Example of an endothermic peak resulting from vaporization of water, obtained by heating a sample to 200 C.

Temperature, °C
170
160
140
120
100
80
60
40
20
mcal/sec

Some samples were allowed to equilibrate at 4 C after preparation for varying periods of time to see whether this affected the results. This was done for both bound water and heat of vaporization measurements.

Weights

A Cahn electrobalance was used for all weighings. Samples were weighed to the nearest 10 micrograms.

RESULTS AND DISCUSSION

Bound Water

Initial results

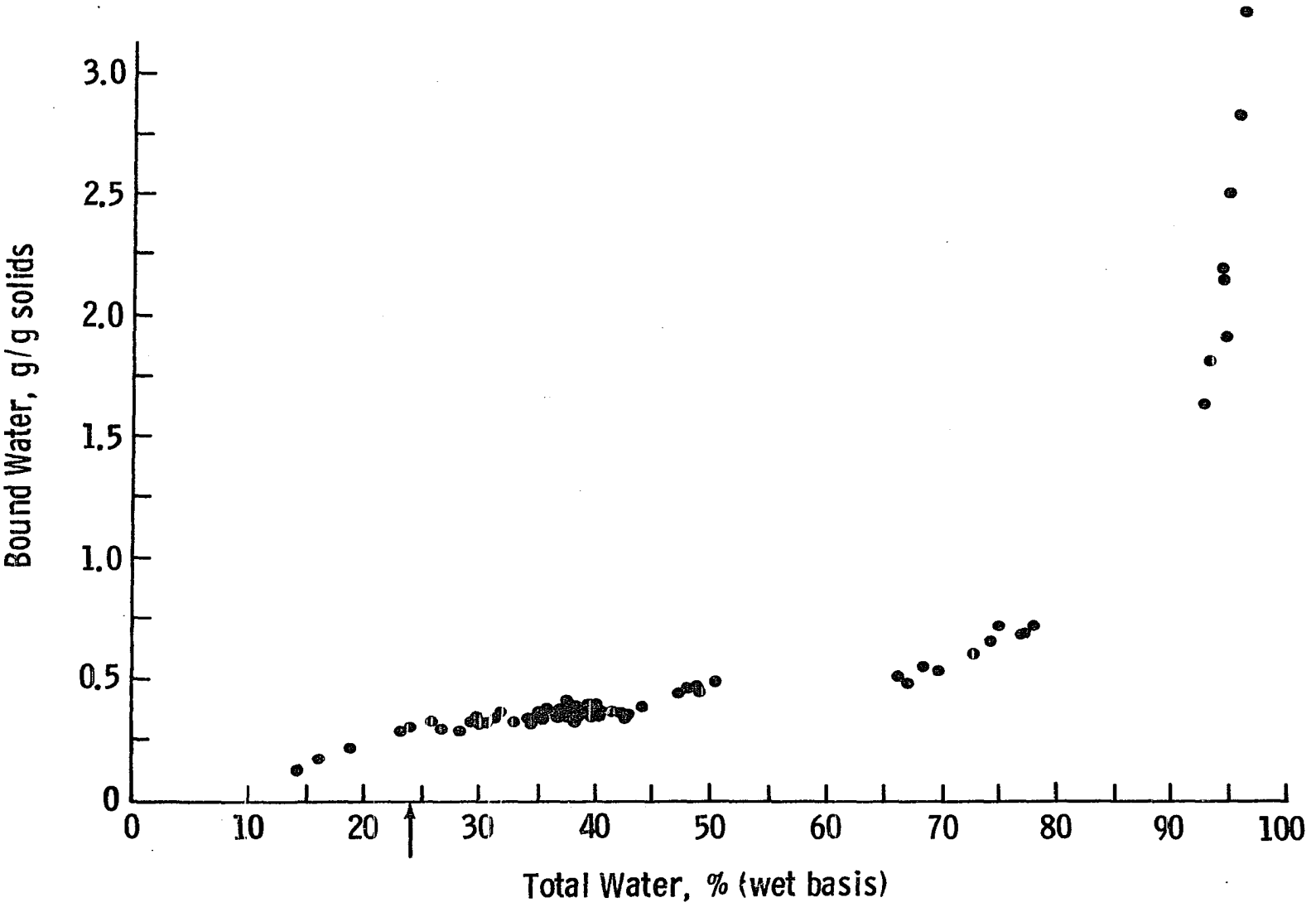
This study was begun with the assumption that the amount of bound water was independent of total moisture content above the "critical" moisture content where free water first appears. But the data that were obtained conflicted with this assumption. They showed that the amount of bound water increased with increasing water content. An example of such data is shown in Figure 7, in which the amount of bound water in Edipro N is graphed as a function of total water content.

Comparison with other bound water studies

The finding that the amount of bound water increased as water content increased was in agreement with the conclusions of some published studies and in conflict with the conclusions of others. Some researchers had concluded that the amount of bound water increases as water content increases. Others had concluded that all water above the critical point is free water, meaning that the amount of bound water is constant above this moisture content.

As has been discussed, all of the researchers who have used NMR to measure bound water have concluded that the amount of bound water is independent of total water

Figure 7. Bound water content of Edipro N as a function of total water content (percent). The arrow indicates the critical moisture content.


Bound Water, g/g solids
3.0
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
40
50
60
70
80
90
100
Total Water, % (wet basis)

content. Researchers who have used calorimetry to measure bound water have concluded either that bound water increases with increasing water content or that it is independent of water content.

Results of other calorimetric studies Ross (1978), Biswas et al. (1975), and Bushuk and Mehrotra (1977) all concluded using DTA and DSC that water binding does increase with increasing water content.

The range of water contents analyzed in other studies was very narrow, so that no conclusions should have been drawn about the relationship between water content and water binding. Berlin et al. (1973) studied whey protein systems with water contents somewhere between 65 and 75 percent water, but they did not actually measure the water contents of individual samples. The bound water values ranged from 0.45-0.52 g/g protein. It was assumed, but not supported with data, that this amount of bound water would not change with water content.

Berlin et al. (1970) studied protein systems with water contents which varied by only a few percent. Hence the fact that the amounts of bound water were constant does not support the idea that the amount of bound water is the same at all water contents. In spite of the lack of supportive data, the existence of this constant level of bound water was assumed by the authors in their discussion.

Ruegg et al. (1974) stated that the water contents of

their casein samples varied from 0.3 to 5.0 g/g, but data for only one water content were presented. They did state that the amount of bound water did not vary with moisture content, but data to support this contention were not presented. Hence their statement is less than convincing.

Duckworth (1971) concluded that the water content where free water first appeared was the maximum amount of water a food would bind and that any additional water was free water. However, the actual amounts of free and bound water were not measured. His conclusion was an assumption unsupported by data.

Measurement of bound water in other protein systems

The conflict in the literature, coupled with the initial data that showed a definite pattern of increasing bound water contents, suggested that additional study of the relationship between bound water content and total water content was necessary.

Other protein systems were studied to determine whether the increase in bound water with increasing moisture content was unique to one protein system or a property of all protein systems.

The bound water contents of Supro 610, ovalbumin, soy protein concentrate (SPC), soy flour, and Edipro A were measured at different water contents. Figures 8 and 9 show the bound water content (g/g solids) graphed as a

Figure 8. Bound water content of SPC as a function of total water content (percent).

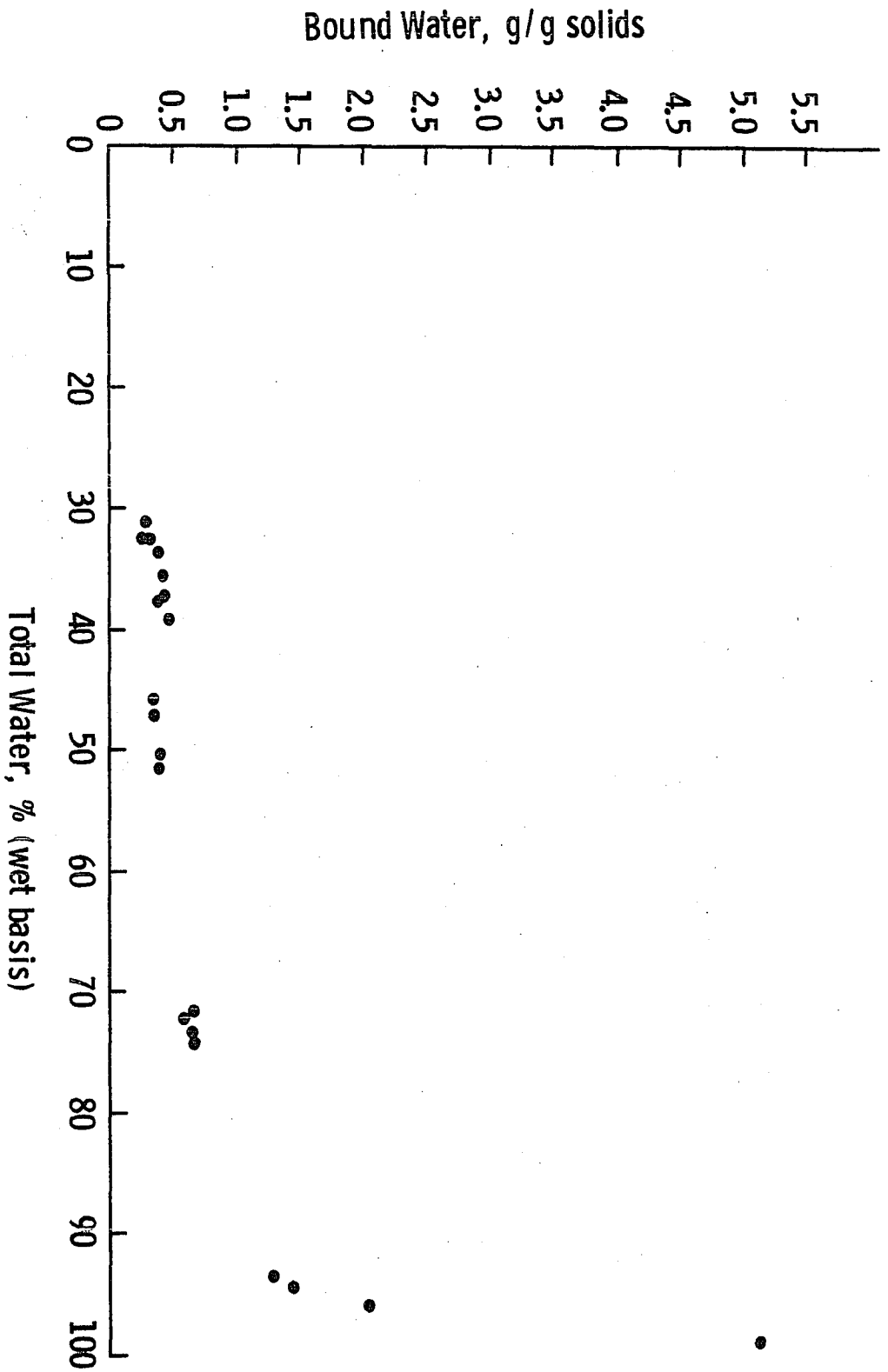
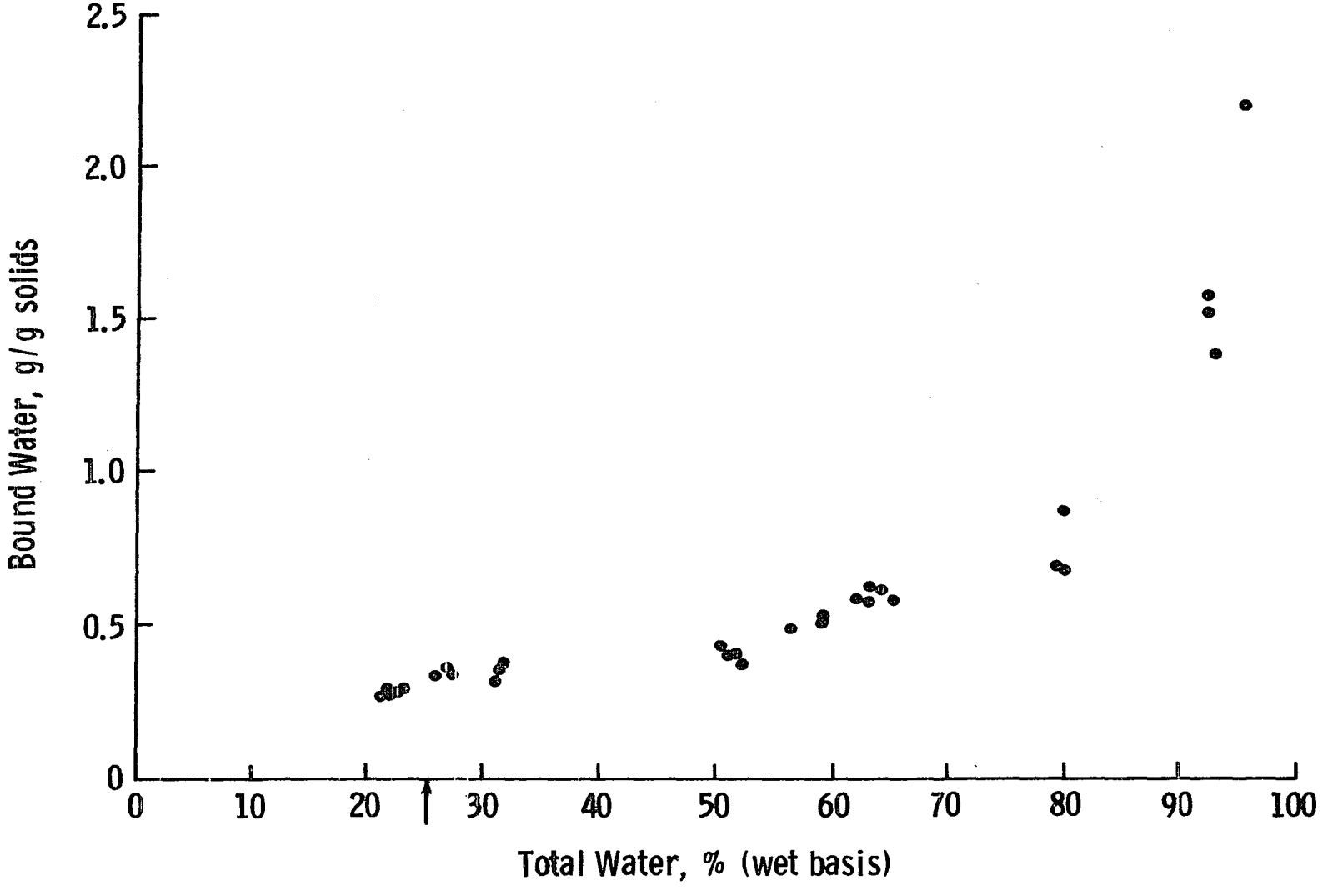

Bound Water, g/g solids
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
Total Water, % (wet basis)
0
10
20
30
40
50
60
70
80
90
100

Figure 9. Bound water content of ovalbumin as a function of total water content (percent). The arrow indicates the critical moisture content.


Bound Water, g/g solids
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
40
50
60
70
80
90
100
Total Water, % (wet basis)

function of total water content (expressed as percent of wet weight) for SPC and ovalbumin, respectively. The data for all of the protein systems showed an increasing amount of bound water per gram solids with increasing water content.

Comparison of bound and free water contents

When bound water content was graphed as function of percent water, as in Figures 7-9, the rate of increase of bound water levels appeared to increase with increasing water content. Further analysis of the data, however, showed that Figures 7-9 were misleading in this respect.

When total water content was expressed as g/g solids, rather than percent of wet weight, graphs of the data for Edipro N, SPC, and ovalbumin suggested a linear relationship between bound water content and total water content. These graphs are shown in Figures 10, 11 and 12, respectively. Expressing both bound and total water content as g/g solids makes possible a direct comparison between the two values, a comparison which reflects the actual physical situation in the hydrated protein.

Similar graphs, with total water content expressed as g/g solids, were prepared for the Supro 610, Edipro A, and soy flour data. These graphs, shown in Figures 13, 14, and 15, respectively, also suggested a linear relationship

Figure 10. Bound water content of Edipro N as a function of total water content (g/g solids).

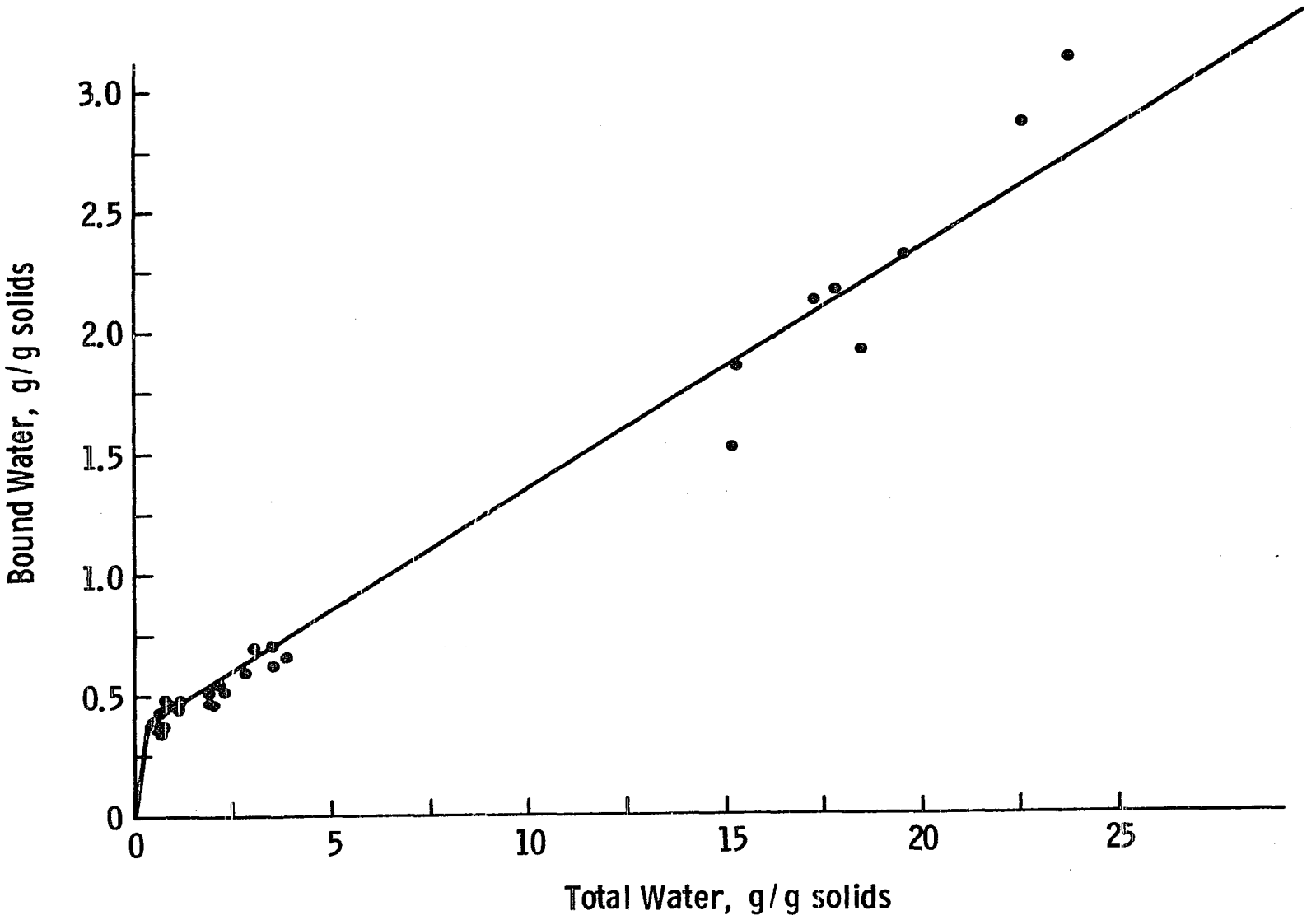

Bound Water, g/g solids
3.0
2.5
2.0
1.5
1.0
0.5
0
0
5
10
15
20
25
Total Water, g/g solids

Figure 11. Bound water content of SPC as a function of total water content (g/g solids).

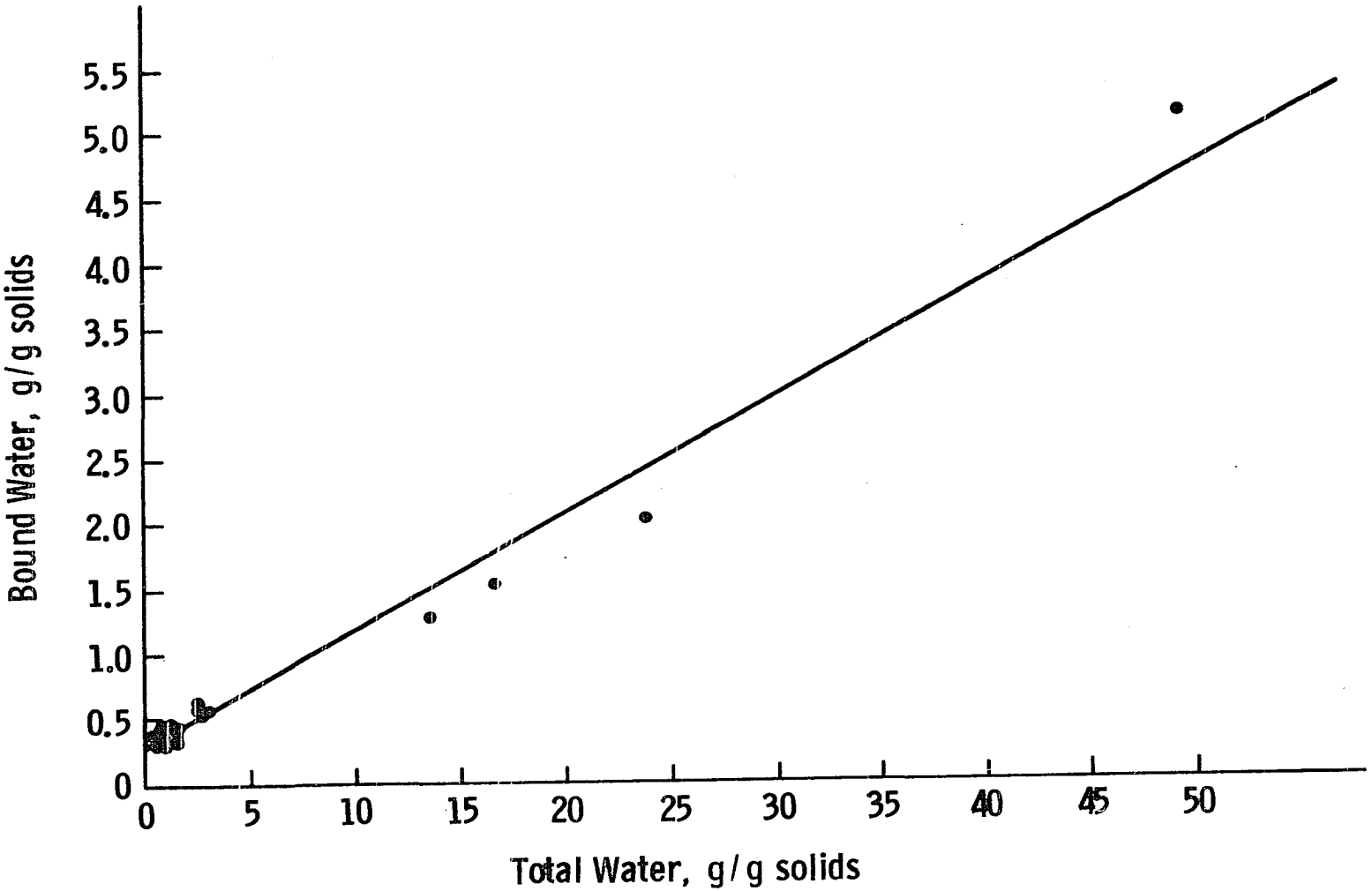

Bound Water, g/g solids
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
5
10
15
20
25
30
35
40
45
50
Total Water, g/g solids

Figure 12. Bound water content of ovalbumin as a function of total water content (g/g solids).

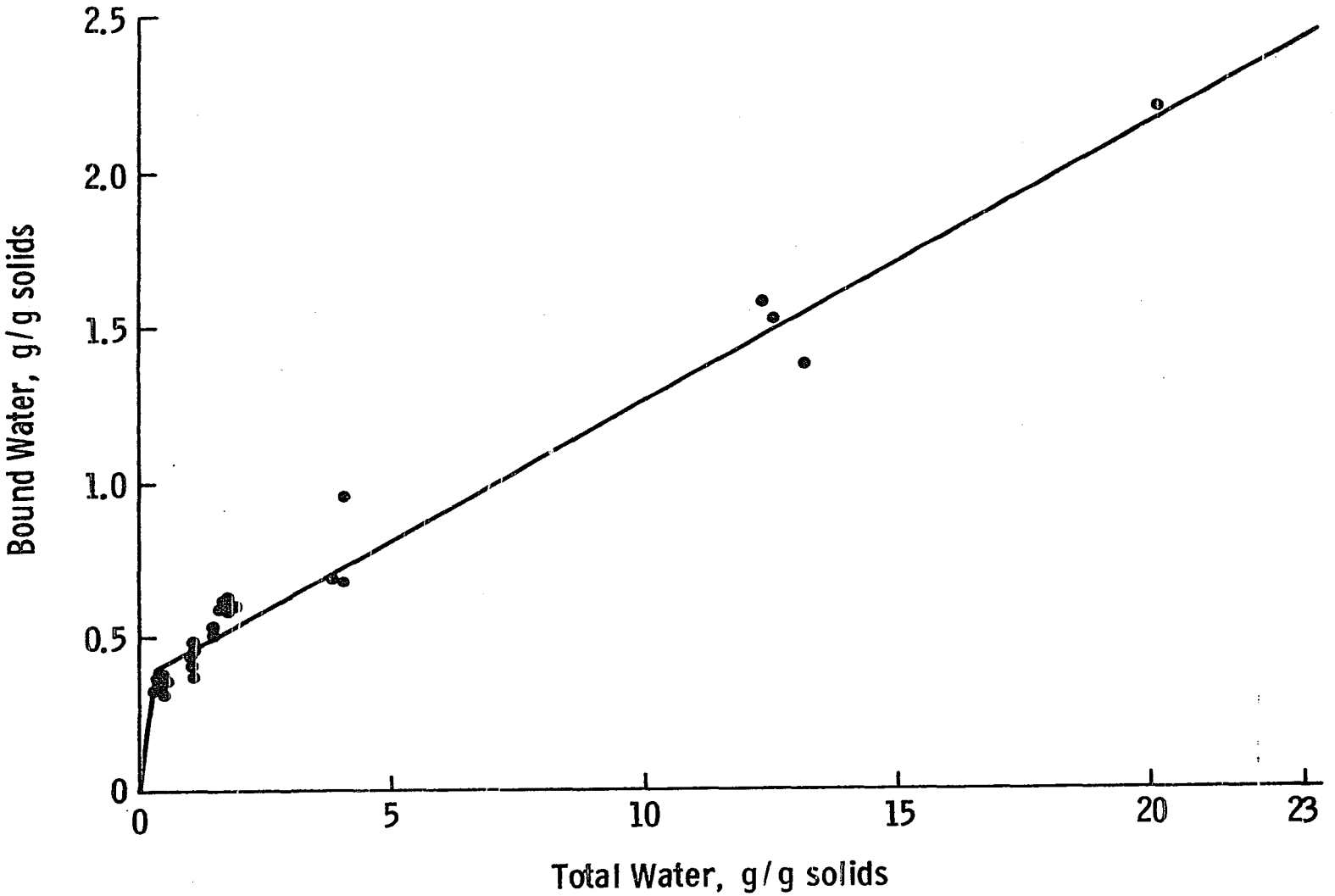

2.5
2.0
1.5
1.0
0.5
0
Bound Water, g/g solids
0
5
10
15
20
23
Total Water, g/g solids

Figure 13. Bound water content of Supro 610 as a function of total water content (g/g solids). Equilibrated samples were held at 4 C for 7 days before analysis.

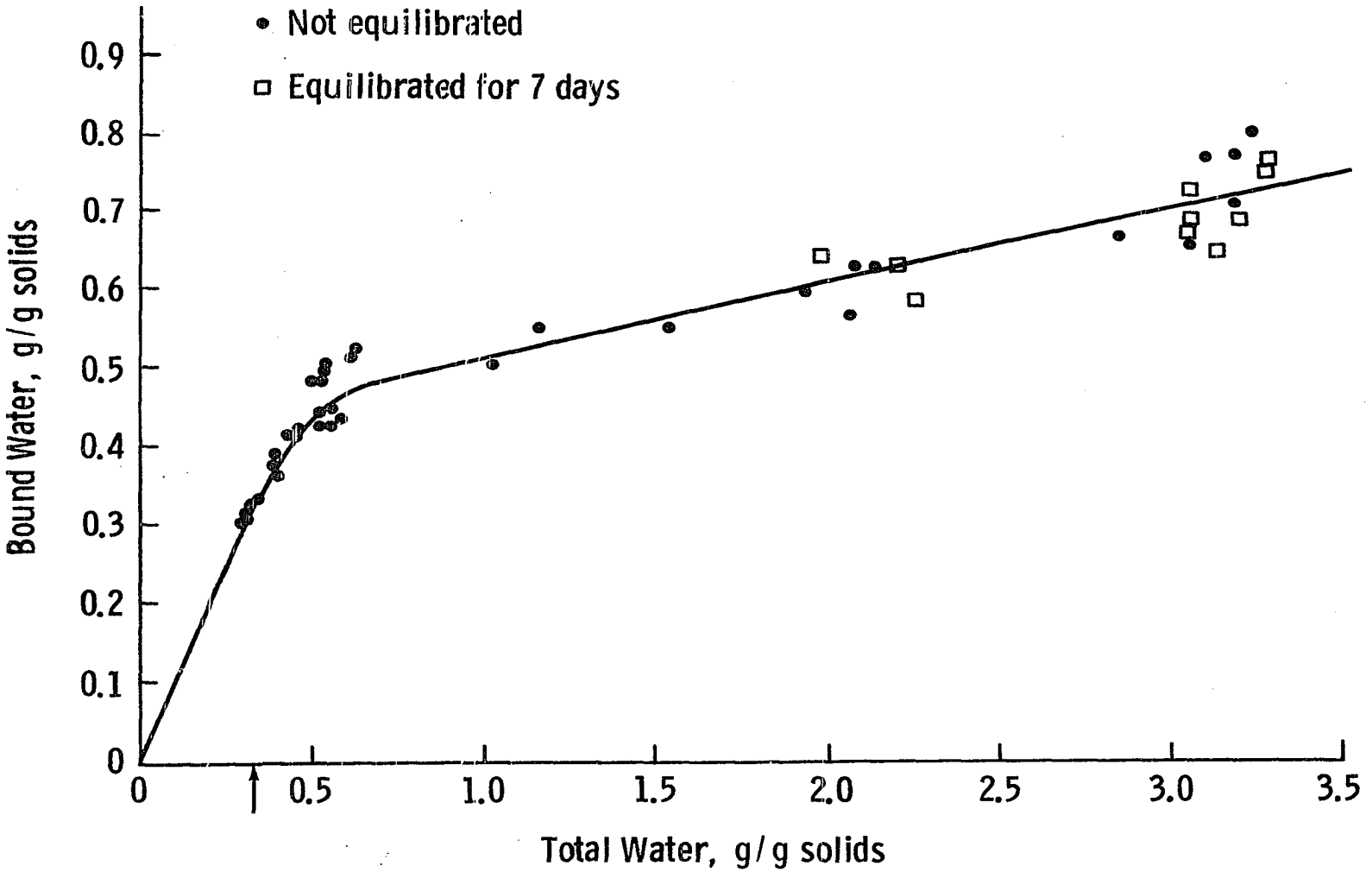

• Not equilibrated
□ Equilibrated for 7 days
Bound Water, g/g solids
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Total Water, g/g solids

Figure 14. Bound water content of Edipro A as a function of total water content (g/g solids).

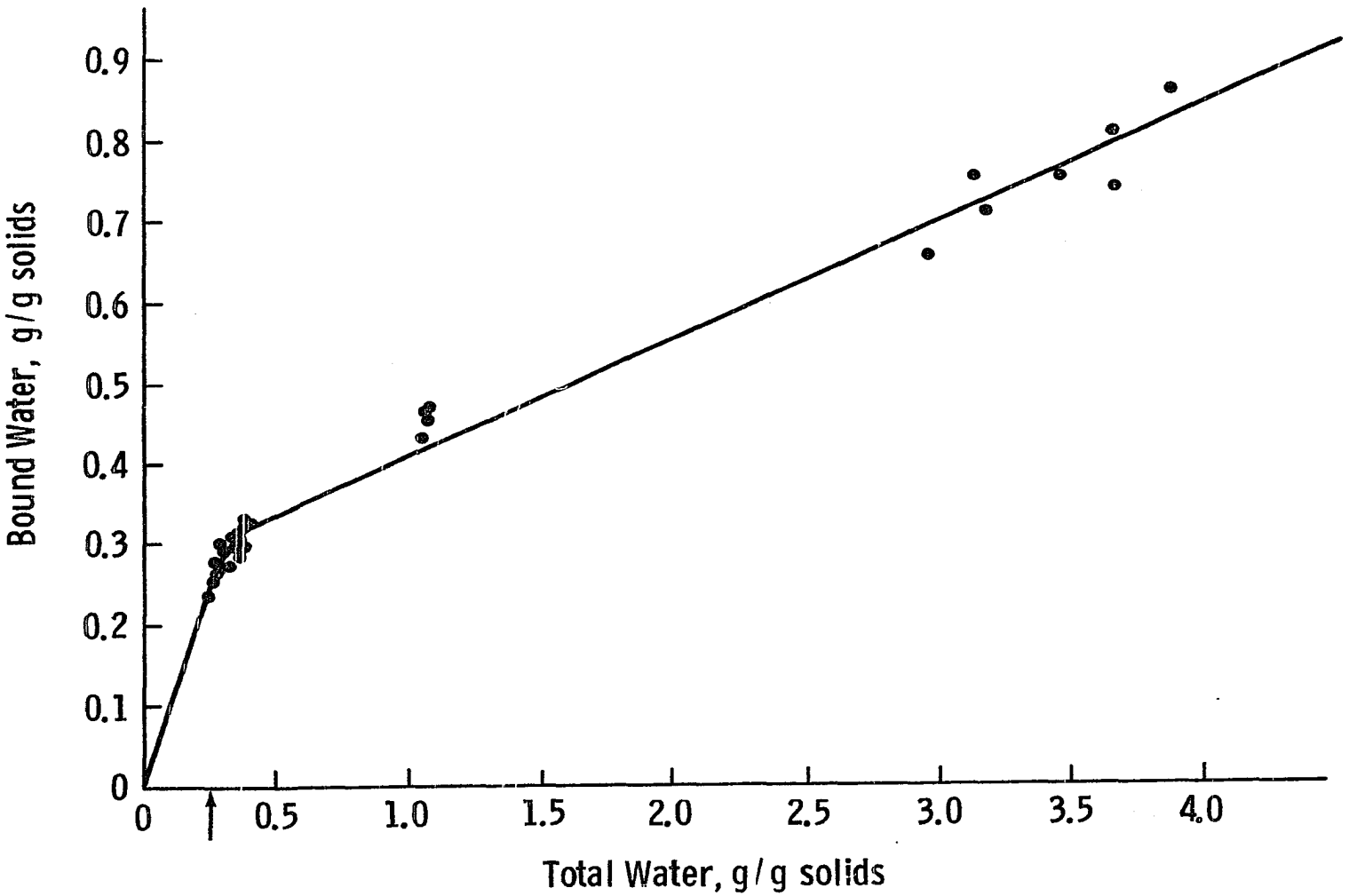
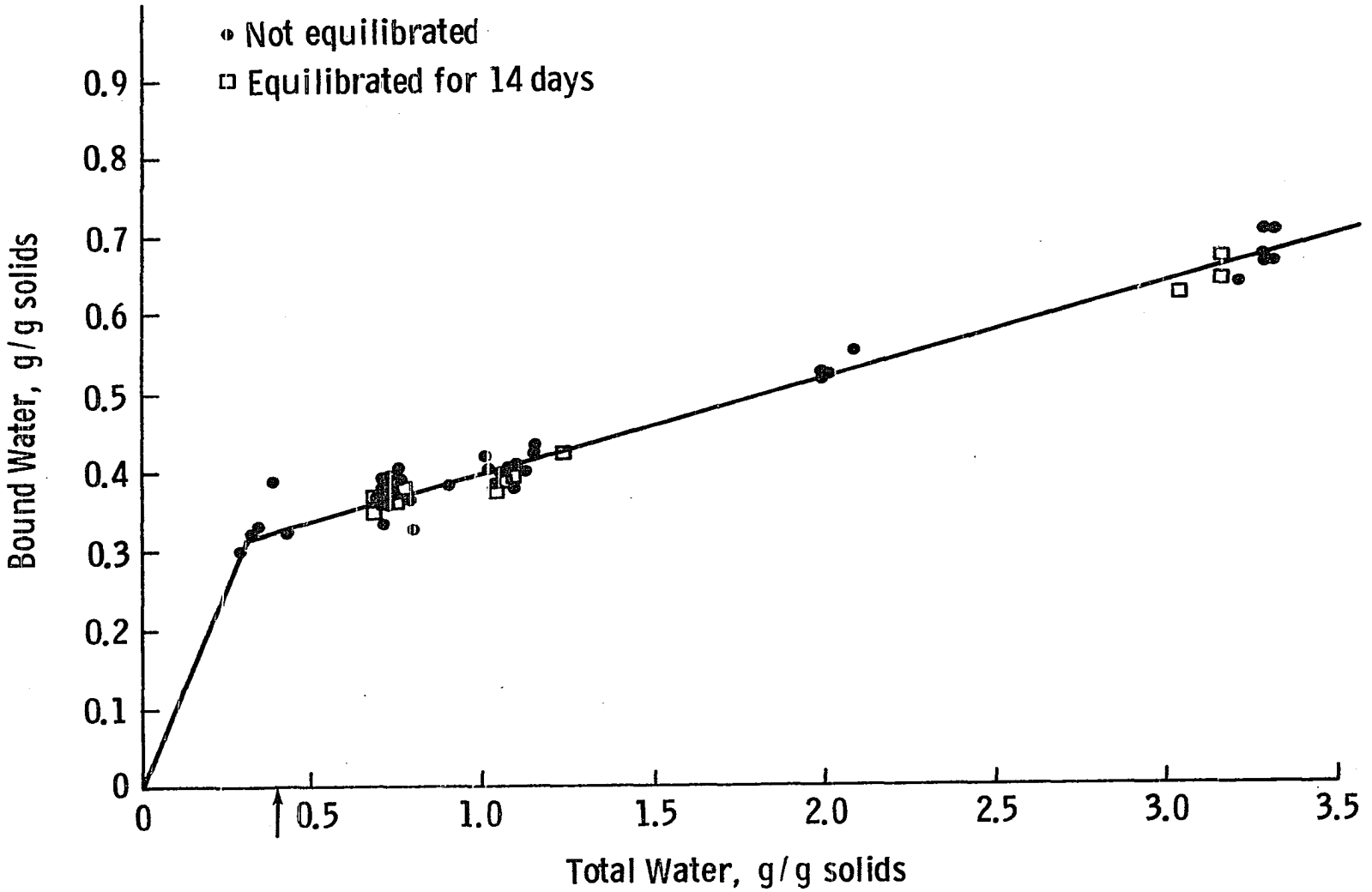

Bound Water, g/g solids
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Total Water, g/g solids

Figure 15. Bound water content of soy flour as a function of total water content (g/g solids). Equilibrated samples were held at 4 C for 14 days before analysis.


Not equilibrated
Equilibrated for 14 days
Bound Water, g/g solids
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Total Water, g/g solids

between bound and total water content.

Linear regression The slopes of Figures 10-15 were calculated using linear regression of Y (bound water, g/g solids) on X (total water, g/g solids). The regression analysis for Supro 610 and Edipro N included data for systems with water contents above 0.5 g/g. The analysis for Edipro A included data for systems with water contents above 0.30 g/g and those for SPC, soy flour, and ovalbumin included all data for systems with water contents above the critical point.

Two separate regression analyses of the ovalbumin, Edipro N, and SPC data were done. The first included only data for systems containing up to 80 percent water; the second included data for the systems containing over 90 percent water as well. To determine whether each slope was significantly different from zero, an F test was used. The calculated slopes and their statistical significance are shown in Table 1.

Table 1. Slopes calculated using linear regression of bound water (g/g solids) on water content (g/g solids)

	For samples that contained up to 80 percent water	For all Samples
Edipro N	0.111 ***	0.104 ***
SPC	0 104 **	0.091 ***
Ovalbumin	0.116 *	0.090 ***
Supro 610	0.096 **	—
Edipro A	0.145 ***	—
Soy flour	0.120 *	—

Significance of the difference of the slope from zero:
*0.05
**0.01
***0.005

All of the slopes, except for that of Edipro A, are roughtly the same, about 0.10. The slopes calculated using the data for systems containing over 90 percent water are essentially the same as those calculated without this high moisture data. This fact, coupled with the high statistical significance of the slopes, means that the actual increase in bound water with increasing water content must be roughly linear. Thus, the rate does not increase with increasing water content, as Figures 7-9 make it appear.

These data can be interpreted to mean that, as water is added to the systems, about 10-11 percent of this water becomes unfreezable water, and about 89-90 percent of this water remains freezable (free) water. The similarity of the two calculated slopes of the ovalbumin, Edipro N, and SPC systems indicates that these percentages are about the same at high and low moisture contents.

Critical moisture contents

Table 2 shows the lowest water levels where small endothermic peaks indicating free water first appeared. Below these moisture levels all of the water was bound. These levels will henceforth be called the critical moisture contents.

The critical moisture content of Edipro A is lower than those of the other systems. Edipro A, with a pH of 4.5, is

the only one of the proteins that is at its isoelectric point.

Table 2. Critical moisture contents

Protein	Total water g/g solids	Total water percent, wet basis
Edipro N	0.32	24.0
Ovalbumin	0.33	25.0
Supro 610	0.33	25.0
Edipro A	0.26	20.6
Soy flour	0.41	29.0

At this pH, some of the potential water binding sites interact with each other, and thus are unavailable to hydrogen bond with water. Hence it is not surprising that less water binds to Edipro A than to the other proteins before free water first appears.

The critical moisture content of soy flour is higher than those of the other systems. Soy flour is the only system studied that contains a significant amount (about 30 percent) of carbohydrate. Since carbohydrates, with their numerous hydroxyl groups, contain more water binding sites per gram than do proteins, one would expect more water to bind to soy flour than to the pure proteins before the appearance of free water.

A line drawn through the group of points just above the critical point on Figures 13 and 14 would have a slope of

about 0.50, meaning that 50 percent of all water added in this region becomes bound. These points on the Supro 610 graph (Figure 13) represent samples containing 0.33-0.65 g water/ g solids, and the actual slope calculated using linear regression is 0.53. On the Edipro A graph (Figure 14), they represent samples containing 0.26-0.40 g water/g solids, and the actual slope is 0.46.

This slope of about 0.50 is much larger than the slope of 0.145 that was calculated for Edipro A. It is also larger than the overall slope of 0.096 that was calculated for Supro 610 using only points for samples containing over 0.5 g water/ g solids.

Therefore, the moisture contents at which the slopes become 0.10 are above the critical moisture levels, at least for Supro 610 and Edipro A. These higher, "significant" moisture levels mark the points above which about 10 percent of all added water consistently becomes "bound" and about 90 percent remains free. Because fewer data were obtained at these moisture contents for the other protein systems, it is impossible to definitely conclude that they have comparable significant moisture levels above the critical moisture levels. However, there is no reason to believe that they do not.

Methodological variables

Since the data in this study did conflict with many published conclusions about water binding, it was necessary

to be sure that the observed increase in water binding was a real phenomenon and not an apparent one caused by an experimental variable. For this reason, four aspects of the analytical procedure were varied.

Some of the Supro 610 and soy flour samples were allowed to equilibrate at 4 C for 7 or 14 days before analysis. As Figures 13 and 15 show, this equilibration following preparation did not alter the amount of bound water present. Also varied were the freezing rate, the heating rate, and the time for which the samples were held at -30 C before heating. None of these variations had any effect on the results, hence there is no evidence that the observed increase in water binding with increasing water content was an artafact and not a real phenomenon.

Conflict with NMR data

There is a conflict between the conclusions about water binding that have been drawn from NMR data and the results of our study. Many of the researchers who have measured the amount of bound water as a function of water content have concluded that the amount of bound water is constant, regardless of the total water content. Their data are no doubt correct, but the assumptions upon which their conclusions are based may be incorrect.

Kuntz et al. (1969) measured the area of narrow NMR signals obtained from protein solutions to determine the

amount of bound water present. The fact that such narrow signals persisted at subfreezing temperatures was interpreted to mean that the water giving the signal was unfrozen and had retained a high degree of mobility compared to that in ice The narrowness of the signals showed that the water lacked an organized structure, even though its mobility was less than that of bulk water. These conclusions are reasonable; there is no reason to question them.

Kuntz (1971), in a continuation of this work, was able to correlate the amount of this unfrozen, relatively mobile water with the hydration of specific hydrophilic and ionic sites on the constituent amino acids. Hence, the bound water measured by this method must be the water that is hydrogen bonded to these sites on the protein molecules. This is a reasonable assumption.

On the other hand, Kuntz's assumption that the bound water measured in this way constitutes <u>all</u> of the unfreezable water present seems questionable. He presented evidence to show that the water he was measuring was bound. He did <u>not</u> present evidence that this was the only bound water present.

Water could be unfreezable for more than one reason. The water that Kuntz measured is unfreezable because it is tightly bound to specific sites on protein molecules. Perhaps there is another fraction of water that is unfreezable because it is part of a structure which is different from ice, but

yet highly ordered-ordered enough that it does not lose this order and form solid ice when the solution is cooled to sub-freezing temperature.

Berendsen (1962) and Fuller and Brey (1968) hypothesized the formation of such a structured water fraction based on their NMR data from hydrated BSA and collagen. According to their studies, the first water molecules added to a dry protein do hydrogen bond to specific sites on the proteins. This water retains a high degree of rotational mobility. It is free to reorient in ways not possible for water participating in more than one hydrogen bond (Fuller and Brey, 1968). This is the same water fraction that Kuntz described.

According to Berendsen (1962) and Fuller and Brey (1968), a cooperative ordering of some of the water molecules into a more structured form occurs when the water content is high enough. At water contents below this "structuring" level, the water is singly hydrogen bonded directly to polar sites or to the water molecules that are bound to the polar sites (multilayer concept). The molecules which form the structure are participating in two or more hydrogen bonds, not just one as are molecules bound to specific protein sites. This structured fraction is much less mobile than the water initially bound to the hydrophilic and polar sites, and so does <u>not</u> contribute to the area of the narrow signal produced by the more mobile fraction of water (Berendsen, 1962). Thus, the

first hydrogen bonded layers of bound water retain much mobility and produce a narrow signal. As the water content increases, some of the molecules form an organized structure in which the mobility of the molecules is restricted to a degree that precludes their producing narrow NMR signals.

Berendsen (1962) and Fuller and Brey (1968) did not determine whether or not this structured water is unfreezable. But if it is, it is bound water by definition, yet "bound" not because of direct interaction with the protein, but because of multiple interactions with other water molecules. If, as is likely, the water structure forms around the nonpolar side chains, the presence of the protein is making this "binding" possible, even though the primary interactions are between the water molecules only.

Since this structured water does not contribute to the narrow NMR peak, it would not be measured as bound water by Kuntz's method. Kuntz's method would only measure the water molecules bound in the usual sense to specific protein sites.

Considering the molecules which are being measured by Kuntz's method, it is not surprising that the amounts of bound water that he measured were independent of water content. It is only logical that there would be an upper limit to the amount of water that could bind directly or in multilayers via hydrogen bonds to specific protein sites.

The elevation of the "structural" heat capacity of water bound to whey over that of water or ice (Berlin and Kliman, 1974) also supports the idea that there is a fraction of sorbed water that exists in a structured form involving multiple hydrogen bonding. They emphasized the point that the water molecules bound to isolated specific sites on the protein surface would not exhibit this structural heat capacity. Structural heat capacity is attributed to the breakdown of an associated water structure (Berendsen, 1967).

Toledo et al. (1968) measured bound water in essentially the same way as did Kuntz et al. (1969). The wide-line NMR instrument was adjusted so that only water with a high degree of mobility produced a signal. Thus, water in unfrozen samples produced a signal proportional to the total amount of water present. All frozen samples above the critical moisture content produced the same NMR readout. Such a signal in a frozen system would come from unfrozen water that possessed a high degree of mobility, such as the water bound to specific protein sites. And one would expect the same signal from all samples regardless of water content. But these researchers, like Kuntz, assumed that all of the unfreezable water retained such a high degree of mobility. And this is the point that is highly questionable, because of the convincing evidence that there is a fraction of highly

ordered, much less mobile water, in addition to the directly adsorbed water, which may in fact be unfreezable.

The same reasoning as has been applied to the bound water measurements of Kuntz et al. (1969) and Toledo et al. (1968), applies to Hansen's (1976,1978) measurements on soy protein products and ovalbumin. His NMR measurements of bound water were also based on the assumption that unfreezable (bound) water retains a high degree of mobility.

Hansen (1976) discussed in detail the fact that his bound water values were exactly the same as predicted by the Bradley isotherm equation. This equation is based on the adsorption of a polarized multilayer of molecules to specific polar sites on the protein surface, via the orientation of dipole moments induced by the polar surface. This is further evidence that this NMR method of measuring bound water only measured the polarized multilayers bound to specific polar sites, water that all researchers agree retains a high degree of mobility. If there were a fraction of less mobile, yet unfreezable, water in addition to this multilayer, it would not be measured by this NMR method.

The NMR method used by Shanbhag et al. (1970) and Okamura et al. (1978) to measure bound water was based on the fact that the bound water in systems containing 25 percent water or less produced a constant NMR readout per gram of water at an attenuation of 0 db. Free water

produced no signal at this attenuation.

At water contents of 25 percent and below, all water in these food systems (wheat flour, corn starch, egg white) is bound. This is the moisture content range of multilayer adsorption. Thus the data (Shanbhag et al., 1970) do show that the bound water in this moisture content range produces an NMR readout proportional to the amount of bound water present. The data also show that free water unquestionably does not produce a readout at this attenuation.

However, Shanbhag et al. (1970) and Okamura et al. (1978) assumed that the NMR readout at 0 db at water contents above 25 percent represented the entire bound water content. This assumption is not supported by any data and is therefore questionable. If the unfreezable (bound) water fraction in higher-moisture systems does in fact consist of two very different categories of water, there is no reason to think that a characteristic signal produced by one of these categories would also be produced by the other. Yet this is the assumption that was made.

The NMR readout (87 units per gram water) at 0 db from systems with high moisture contents no doubt has exactly the same meaning that it does at the low moisture levels-it represents the amount of water in the multilayer adsorption fraction. If this is true, then it is not surprising

that the readout remains constant as the water content increases above the critical point.

The fact that free water produces no signal at 0 db does not mean that free water is the only water fraction that produces no signal at 0 db. Yet this was assumed. Perhaps there is a fraction of structured, "bound" water which also does not produce a signal at 0 db. Because the properties of this fraction, if it exists, are very different from those of multilayer water, it would not be expected to produce the same signal at 0 db, and might very well not produce any signal at all. If this were the case, this method of measuring bound water would fail to measure this structured fraction, even though it was unfreezable, "bound" water. Even though the NMR data on bound water seem to show that the amount of bound water is independent of water content, it is likely that this conclusion is based on false assumptions. These NMR methods are probably measuring only the amount of multilayer bound water, which would be independent of moisture content. Any structured "bound" water, if it exists, is not being measured by these methods. And so, if the amount of this structured bound water did increase with increasing moisture content, this increase would not be measured by the NMR methods that have been used to measure bound water. Thus, these NMR papers on bound water do not prove or disprove

the idea that the amount of bound water may increase with increasing moisture content. They do not provide evidence for challenging the calorimetric data that show that the amount of unfreezable water does increase with increasing moisture content.

Heat of Vaporization

To gain more information about bound water, the average energy required to remove water from these protein systems at different water contents was measured.

Results

Figures 16, 17 and 18 show the heat of vaporization (ΔH_v) graphed as a function of water content for Supro 610, Edipro N, and ovalbumin, respectively. This ΔH_v is an average (integral) value. The heat of vaporization of all of the water molecules is not the same. So the integral ΔH_v is determined by the amounts of water molecules with different heats of vaporization.

The graphs in Figures 16-18 have a common pattern of change in ΔH_v with increasing water content. At the lowest water contents the ΔH_v is high. Then the ΔH_v declines dramatically, reaching a minimum at about 0.14-0.19 g water/ g solids (13-16 percent water). Above this water content, the ΔH_v increases by about 180-230 cal/g, reaching a maximum at 0.21-0.26 g water/ g solids (17.4-20.6 percent water).

Figure 16. Average heat of vaporization of water from Supro 610 as a function of water content. The arrow indicates the critical moisture content.

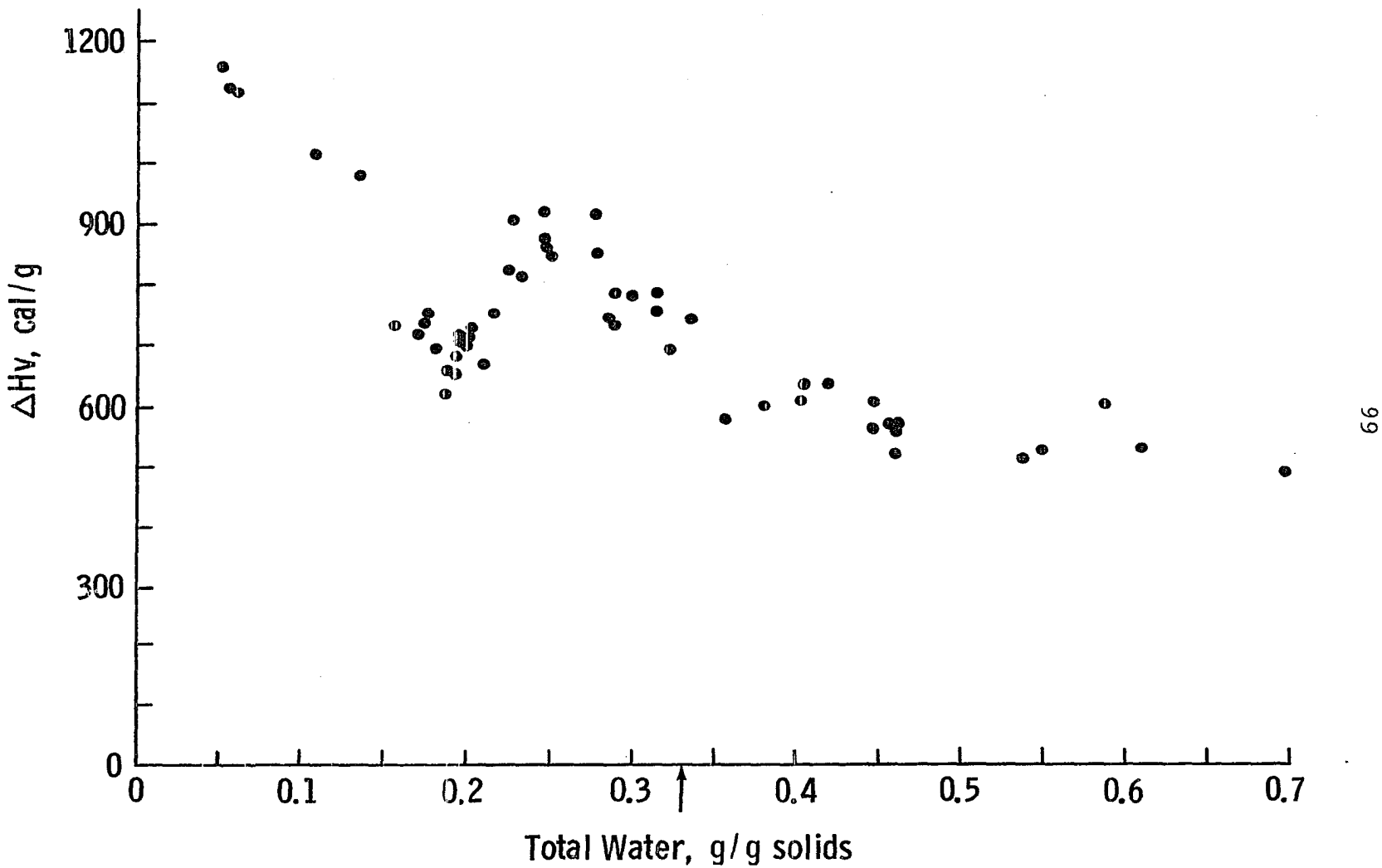

1200
900
600
300
0
ΔHv, cal/g
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Total Water, g/g solids

Figure 17. Average heat of vaporization of water from Edipro N as a function of water content. The arrow indicates the critical moisture content.

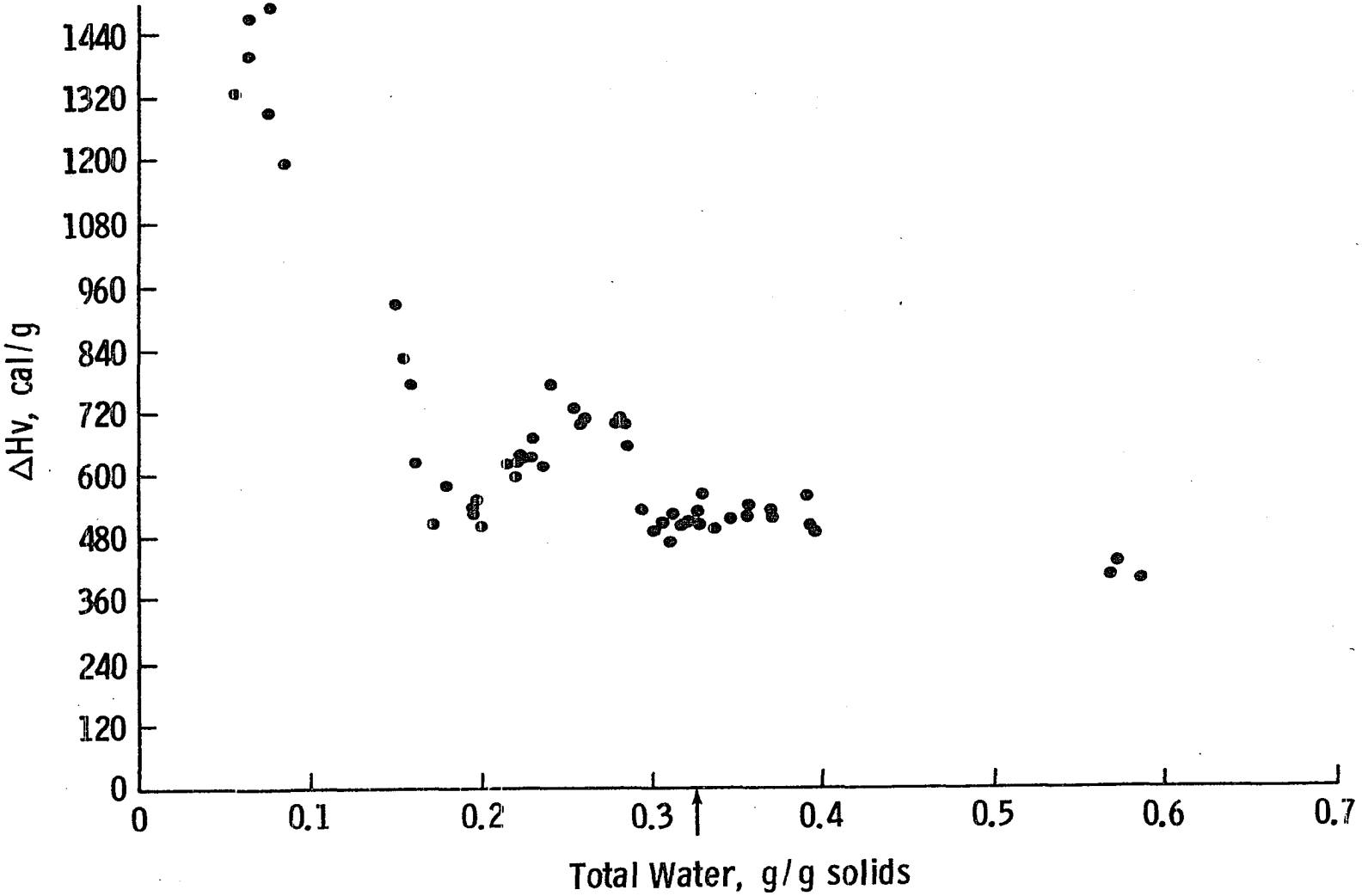
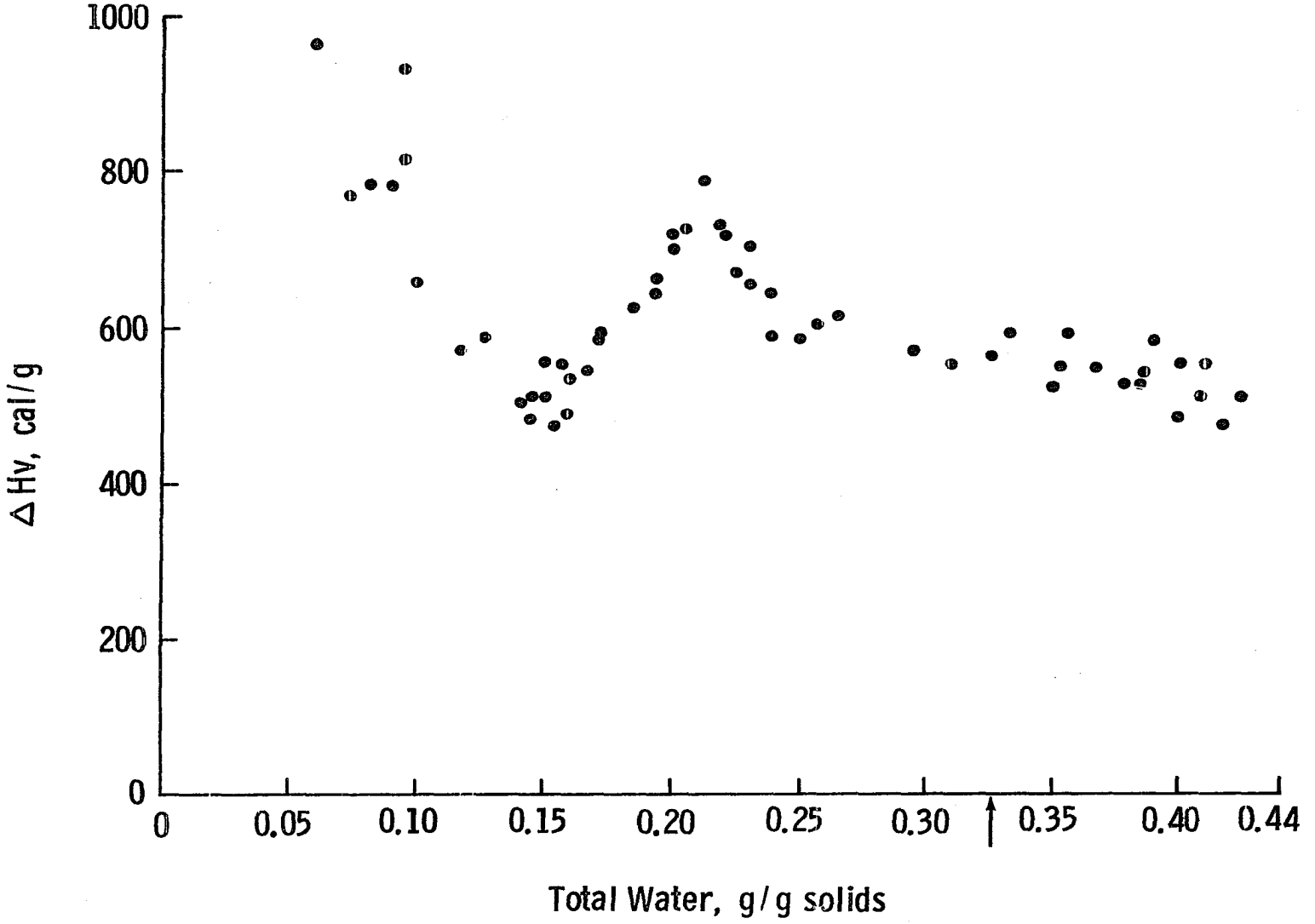

1440
1320
1200
1080
960
840
720
600
480
360
240
120
0
ΔHv, cal/g
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Total Water, g/g solids

Figure 18. Average heat of vaporization of water from ovalbumin as a function of water content. The arrow indicates the critical moisture content.


1000
800
600
400
200
0
ΔHv, cal/g
0
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.44
Total Water, g/g solids

As the water content increases further, the ΔH_v declines again.

At 0.45 g water/g solids, the ΔH_v of water from ovalbumin and Edipro N had declined to about 500 cal/g. With increasing water content, the ΔH_v approached that of pure water, which was found to be 450 cal/g. Edipro N systems containing 2.85 g water/g solids (74 percent water) had an average ΔH_v of 472 cal/g. The ΔH_v of water from Supro 610 containing 0.45 g water/ g solids was about 550 cal/g. The average ΔH_v of water from Supro 610 containing 0.92 g water/ g solids (40 percent) was 463 cal/g, very near that of pure water. The ΔH_v for Supro 610 systems with 3.0 g water/ g solids (75 percent water) was indistinguishable from that of pure water.

Although Figures 16-18 do show a common pattern of change in ΔH_v as water content increases, there are two kinds of differences between them. The water content at which the ΔH_v increases after the initial decline is lower in ovalbumin than in Edipro N or Supro 610. And secondly, the magnitudes of the ΔH_v at the initial minimum and the subsequent peak are higher in Supro 610 than in Edipro N or ovalbumin. These data are summarized in Table 3.

Table 3. Water contents and ΔH_v values associated with the increase in heat of vaporization

	Minimum point (before increase)		Maximum point		Difference in ΔH_v between minimum and maximum points (cal/g)
	Water content (g/g solids)	ΔH_v (cal/g)	Water content (g/g solids)	ΔH_v (cal/g)	
Supro 610	0.19	660	0.25	875	215
Edipro N	0.19	525	0.26	725	200
Ovalbumin	0.15	510	0.21	750	230

Analysis of results

The ΔH_v data from Edipro N, Supro 610, and ovalbumin clearly show that there is an increase in ΔH_v at about 20-25 percent water. To interpret the meaning of this increase, the cause of the increase must be known. The heat of vaporization is the energy required to overcome the energy of the bonds holding the water in the food and thus to remove the water from the food. There are two reasons the ΔH_v might increase. There could be an increase in the energy of the individual bonds involved, or there could be an increase in the number of bonds holding each water molecule in the food system.

There is no reason to believe that the bonding strength increases. It is generally accepted that physical adsorption is accompanied by a decreasing heat of adsorption with increasing surface coverage (Berlin et al., 1970; Hansen, 1976). And this fact is substantiated by the rapid initial decline in ΔH_v that occurred as water was added to the dry protein systems.

Thus, the second reason for an increase in ΔH_v seems the most likely - an increase in the number of hydrogen bonds in which the water molecules are participating. This

is the idea that was proposed by Berlin et al. (1970) to explain the increase in ΔH_v which they observed at a water content above 0.18 g/g solids--a cooperative ordering of water molecules to form a structure in which water molecules have a vastly reduced degree of mobility because of their participation in multiple hydrogen bonds.

Below the water content where the ΔH_v increases, the water molecules are participating in only one hydrogen bond, with either a polar food site or one other water molecule (Berlin et al., 1970; Fuller and Brey, 1968). The heat of vaporization must overcome the energy of this one bond. If, with sufficient water coverage, some of the water molecules interact with each other to form an organized structure, the heat of vaporization would have to overcome the energy of two to four hydrogen bonds per water molecule, instead of only one. This would account for the increase in ΔH_v.

The energy of one hydrogen bond is 3-6 kcal/mole or 167-333 cal/g (Fennema, 1976). The increases in ΔH_v at 0.21-0.26 g water/g solids that were measured in this study, 200-230 cal/g, fall within this range. This suggests that there is an average increase of one hydrogen bond per water molecule when the structured water of hydration forms.

The fact that the ΔH_v of this water is higher than that of pure water, coupled with the NMR evidence showing that it is much less mobile than pure water (Berendsen, 1962; Fuller and Brey, 1968), means that it has a structure that is different in some way than the structure of bulk water. As has been discussed, most researchers agree that bulk water also has an organized structure of some kind. The best-accepted theory of the structure of bulk water (Nemethy and Scheraga, 1962) suggests that bulk water consists of a mixture of water clusters and nonhydrogen bonded single water molecules. The clusters consist of water molecules that are participating in one to four hydrogen bonds. The water molecules in the interior of the clusters are participating in four hydrogen bonds with adjacent water molecules.

The water structure that forms at a water content of 20-25 percent must contain a higher percentage of molecules participating in four hydrogen bonds than does bulk water. This higher percentage of tetra-hydrogen bonded molecules would account for the greater heat of vaporization and lesser mobility. The nature of this structure is a matter for speculation. It is possible that this structure consists of a higher proportion of clusters than does bulk water, which would imply a higher proportion of tetra-hydrogen bonded water molecules. Perhaps the equilibrium that exists in bulk water is shifted in the direction of more clusters and fewer

unbonded molecules.

Frank and Quist (1961) stated that the addition of nonpolar solutes causes a shifting of the water structure in the direction of greater "ice-likeness", so that some of the monomeric water forms a new framework, or structure. It is likely that the nonpolar sidechains of proteins would have the same effect. Nemethy and Scheraga (1962a) also stated that nonpolar solutes cause the equilibrium in water to shift in the direction of greater ice-likeness, as evidenced by the strongly negative excess entropy and high heat capacity of such solutions. According to these researchers (Nemethy and Scheraga, 1962b), the amount of hydrogen bonding in the vicinity of nonpolar solutes is increased over its average value in pure water. The hydrogen-bonded clusters extend around part of the solute molecules, resulting in the formation of incomplete "cages" around the nonpolar solutes. It is suggested (Nemethy and Scheraga, 1962b) that this same phenomenon occurs with the nonpolar side chains of proteins.

Klotz (1958) said that nonpolar side chains of proteins would be expected to induce a crystalline, cagelike arrangement of hydration water, with the added possibility of long-range cooperative effects due to the presence of many such side chains bound to the frames of the protein molecules. In addition, Klotz (1958) stated that the formation of such

structured hydration water around polar sites was unlikely, since the electric field would be sufficient to disrupt any organized lattice structure.

From these researchers' ideas, a concept of protein hydration emerges. The initial water molecules added to a dry protein bind to polar or ionic sites via single hydrogen bonds. Additional water molecules may bind to these first ones via single hydrogen bonds. When the water content increases to a sufficient level, another type of hydration also begins to occur-a highly ordered, relatively immobile type of water structure forms around nonpolar side chains of proteins. Thus, at least at low water contents, the two types of hydration involve different side chains, and could be occurring simultaneously. There is direct water binding to polar groups, and a structuring of water around nonpolar groups. At higher water contents, it is possible that structured hydration water could form and essentially surround the entire protein. This would be possible as water contents increased because hydration shells around nonpolar groups would be close enough to each other to interact, as Klotz (1958) suggested.

Relationship to NMR data

Kuntz et al. (1969) found the bound water content of ovalbumin solutions containing 95 percent water to be 0.31

g/g protein. Arguments have been presented which strongly suggest that Kuntz's NMR method only measured water bound to polar sites and did not measure structured "bound" water. The fact that the ovalbumin data in this study show an increase in H_v at 0.20 g water/ g protein because of the formation of structured bound water does not refute these arguments.

There is no reason to believe that multilayer binding of water to polar sites suddenly ends when the formation of structured water of hydration around nonpolar groups begins. Since differenct side chains are involved, both types of hydration could and very likely do proceed simultaneously at the low moisture levels where the structured bound water begins to form. Thus, the fact that the H_v peak occurs at a water content below Kuntz's bound water value of 0.31 g/g protein does not mean that Kuntz's measurement included the structured bound water. It simply means that 0.31 g/g protein of multilayer bound water was present along with some structured bound water.

This idea of both types of hydration occurring simultaneously at low water contents may be related to the "significant" moisture level discussed earlier. This significant moisture level is the water content above which 10 percent of added water consistently becomes bound and 90 percent remains free. Between the critical moisture content

and the higher significant moisture content, about 50 percent of added water becomes bound. It is possible that between the critical and significant levels both types of hydration are occurring. Since the lower and constant slope of 0.10 begins at the "significant" level, multilayer adsorption must cease at this point. At water contents above this level, the water which becomes bound is probably structured bound water.

Theory About Unfreezable Water

Table 1 shows the lowest water contents where some free water exists in the protein systems studied. Below these water contents, all water is unfreezable (bound). Above these water contents, part of the water is freezable (free) and part of it is bound.

The most crucial observation that one can make from the data in this study is that these critical moisture contents are all well above the water contents where the large increase in ΔH_v occurs. Therefore, if one accepts the idea that some type of water structure forms, these data show that this structured water <u>is</u> unfreezable water. The increase in ΔH_v occurs at water levels where all water is bound, according to the data from this study, as well as data from other studies (Hansen, 1976; Hansen, 1978; Kuntz et al., 1969; Duckworth, 1971; Shanbhag et al. 1970). The fact that

this fraction of structured water is unfreezable and thus is measured as "bound" water by calorimetric methods, is very significant and suggests a theory which may explain how the bound (unfreezable) water content of a protein system could increase with increasing water content.

Crucial to this theory is the existence of an equilibrium between the unfreezable structured water and the freezable bulk water. The best-accepted models of water structure assume that an equilibrium exists between structured and bulk water (Frank and Quist, 1961; Nemethy and Scheraga, 1962b). If the unfreezable structured water is the result of a shift in equilibrium toward more clusters, then an equilibrium must exist. Hazlewood et al. (1969) in their NMR study of ordered water in skeletal muscle showed that free water added to the muscle (in which all water was ordered) exchanged rapidly with the ordered water.

Given that this structured fraction is "bound" water and that an equilibrium exists between this fraction and the bulk water fraction, it seems likely that part of any additional bulk water added to a system will become part of the structured fraction, in order to maintain the equilibrium. And in fact, the data in this study suggest that this does occur, although there may be another explanation.

The slopes calculated using linear regression (Table 1)

are significantly greater than zero. This means that, as water is added to these systems above their "significant" moisture contents, a constant fraction of this added water becomes part of the highly ordered water of hydration and the rest remains bulk water. The fact that the slopes calculated using the data for systems containing over 90 percent water are about the same as the slopes calculated without those data shows that the same proportion of added water is becoming "bound" water at all of the moisture contents studied. This fact would seem to support the idea that part of the added bulk water joins the more ordered, relatively immobile structure in order to maintain an equilibrium between the two types of water.

SUMMARY

In this study the bound water content of soy proteins and of ovalbumin containing 5-98 percent water (0.05-49.0 g/g solids) was measured. Up to a "critical" water content, about 0.33 g/g solids, all water is bound. Between this critical level and a slightly higher "significant" moisture content (about 0.55 g/g solids), about 50 percent of added water becomes bound water. Linear regression analysis of the points above this "significant" level showed that about 10 percent of all additional water becomes bound and 90 percent remains free. Thus, at all moisture levels between 0.55 and 49.0 g/g solids (35-98 percent) there is a linear relationship between bound water and total water, when both are expressed as g water/g solids. This is a new finding. The few other researchers who have found that the amount of bound water is not constant have not established any mathematical relationship between total water and bound water.

The integral heat of vaporization (ΔH_v) of water from proteins containing 0.05-3.0 g water/ g solids (5-75 percent) was also measured. The ΔH_v was high in dry systems, but it decreased rapidly as water was added. With further addition of water, the ΔH_v increased again, reaching a maximum at about 0.2-0.25 g water/g solids. This increase in ΔH_v very likely results from the formation of a structured type of

hydration water around nonpolar amino acids. The molecules in this fraction of water probably participate in a larger number of hydrogen bonds than do those in bulk water.

The fact that the critical moisture content (about 0.33 g/g solids) is above the moisture content where the ΔH_v peak occurs, means that this structured hydration water is unfreezable and therefore "bound" by definition, even though it is not hydrogen bonded to polar food sites. This unfreezability of structured hydration water is a new concept. There are, therefore, two types of unfreezable water: water that is hydrogen bonded to polar food sites; and structured water of hydration that forms around nonpolar side chains.

The information obtained in this study suggests a probable description of protein hydration. The first water added to a dry protein hydrogen bonds directly to polar and ionic sites. Some of the additional molecules hydrogen bond to the first molecules, resulting in multilayer adsorption. When the water content increases to a sufficient level, a structured fraction of hydration water begins to form around nonpolar amino acids. Up to the "significant" water content, both types of hydration probably occur simultaneously. Above this significant water level, all added water that becomes bound, probably becomes structured hydration water.

The NMR methods that are used to measure bound water

appear to measure only the more mobile fraction of bound water that is hydrogen bonded to polar sites. They apparently do not measure the less mobile, highly structured fraction of bound water that forms around nonpolar side chains. Since it is this structured fraction that becomes larger as water content is increased, the increased amounts of bound water are not detected by the NMR methods.

There must be an equilibrium between the structured hydration water and free water. The fact that a constant proportion of added water, over such a wide range of moisture contents (0.55-49.0 g/g solids), becomes bound by joining the structured fraction, is consistent with the maintenance of this equilibrium. If indeed a portion of any added water joins the structured fraction in order to maintain an equilibrium, this would appear to explain how the amount of bound water can increase with increasing water content, an idea that has been rejected by many researchers and unexplained by those who have supported it.

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