

^1H AND ^2H NMR STUDIES OF WATER IN WORK-FREE WHEAT FLOUR DOUGHS

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INTRODUCTION

In the baking industry, the type of wheat flour employed dictates to a large extent the nature of the final baked product. Breads, for example, are prepared exclusively from hard wheat flour, while biscuits, cookies, and cakes are generally derived from soft wheat flours. A striking difference between hard and soft flour, from a baker's perspective, is their difference in water absorption [1]. Hard wheat flour can generally accommodate its own weight in water, while a soft flour at comparable moisture levels forms a soupy mixture and lacks the appropriate physical properties. In this paper we examine, by ^1H and ^2H NMR methods, the hydration of soft and hard wheat flour doughs in an effort to better understand differences between flour substrates in their interaction with water.

NMR relaxation methods have been employed extensively in efforts to quantitate the interaction of water with macromolecules [2,3]. Not surprisingly, there are differences of opinion regarding the interpretation of NMR results relating to the interaction of water with complex macromolecules and substrates. Multicomponent NMR relaxation behavior has been observed for water associated with many materials, including muscle tissue, [4,5] food products [6-8] and amorphous catalyst substrates [9,10]. Models have been proposed which allow interpretation of complex relaxation decay in terms of discrete water states (bound, weakly bound, free, etc.) and exchange processes. Koenig and coworkers, through NMR dispersion measurements, postulated water need not necessarily be "bound" (i.e. hindered mobility) to macromolecules nor in exchange to account for its relaxation properties [11,12]. Instead they postulated that the relaxation behavior of water in close proximity to large molecules is governed by a

hydrodynamic effect as well as cross relaxation which can significantly shorten the relaxation time and, thus, mask the true correlation time of water. In this model, water senses (is coupled to) the slower motions (longer correlation times) of nearby macromolecules giving rise to a wide dispersion of water motional behavior. Bryant and coworkers have also proposed cross-relaxation as an efficient relaxation mechanism for water closely associated with protein [13,14]. They predict that this protein-associated water has a rotational correlation time on the order of picoseconds, suggesting only a slight loss of motion when in the "bound state". From relaxation studies on small proteins, Fullerton has proposed four specific types of water "states" (i.e., domains of mobility). His predictions, based on proteins of known structure and numbers of specific "adsorption sites", are in remarkably close agreement with experimental NMR data as well as measurements from other physical methods [15,16]. Lillford, in contrast to those who subscribe to physically discrete water states as defined by NMR relaxation brought forth the concept of sample heterogeneity to account for multiexponential proton relaxation behavior [17]. In this model, complex relaxation behavior results when the concentration of "bound sites" is not constant over the volume through which a water molecule diffuses during its relaxation time.

In our studies of water hydration of work-free doughs we base our determination of water compartmentalization solely on multiexponential analysis of spin-spin relaxation behavior. We recognize that this analysis simplifies a very complex system, but it has the advantage of allowing us to label water domains consistent with hydration states in a general sense according to motional freedom. The water states, as defined by multiexponential relaxation decay, can then be monitored as a function of flour type, moisture content, hydration time, and mixing.

The studies reported here are concerned primarily with the characterization of work-free flour-water doughs. By work-free we mean precautions are taken to eliminate the effects of mechanical work (mixing) input on the malleable (semi-solid) dough matrix which may itself have secondary effects on the water hydration.

MATERIALS AND METHODS

Work-Free Dough Sample Preparation

For the preparation of work-free wheat flour doughs at fixed water concentrations [45% and 35%, g water/(g water + g dry flour)] ice was finely powdered in a freezer mill (model 6700, Spex Industries, Inc., Edison, NJ) at liquid nitrogen temperature for a period of five minutes and filtered to particles of less than 100 microns. Wheat flour (obtained from Nabisco Brands, Inc., East Hanover, NJ) was precooled and hand mixed with the ice

powder at -40°C in a cold box (Lehrer Microtome Cryostat model LC-2000, Refrigeration for Sciences, Inc., Island Park, NY). While still at -40°C , component amounts of the ice/flour mixture were adjusted to produce a sample containing 35% or 45% total moisture based on dry weight (i.e., analysis of moisture content by drying at 100°C for 48 hours). The ice/flour mixture was then further milled at liquid nitrogen temperature for two additional minutes to ensure a homogeneous distribution of ice and flour. Separate 5mm o.d. NMR glass sample tubes were then loaded with the ice/flour mixture (100-150 mg each) and stored at -40°C . One hour prior to NMR studies, the ice/flour mixture was thawed at room temperature. Samples prepared in this manner are referred to as "work-free" doughs since no physical mixing of liquid state water and flour takes place.

For preparation of the work-free wheat flour doughs at 35% or 45% D_2O content ($^2\text{H} = \text{D}$) samples of hard wheat flour (ca. 14% moisture) were first placed in a vacuum oven at room temperature (ca. 25°C) for a period of two days. This resulted in a weight loss of nearly 13%, i.e., only 1% of the original H_2O remained. The flour was rehydrated in a desiccator over D_2O (99.8%-Merck Isotopes, Rahway, NJ) for a period of 24 hours and the sample evacuation/drying procedure and subsequent rehydration with D_2O repeated. (This removal of hydration moisture from "as is" hard flour was not employed in the H_2O hydration protocol, vide supra.) The final moisture content of the dough sample after the second rehydration was adjusted to 14% D_2O total moisture (based on dry weight). Further preparation of the ice/flour mixture at 35% or 45% D_2O ice content was carried out as described above except powdered D_2O ice was used instead of H_2O .

In preparation of work-free ice-flour mixtures at 35% and 45% (H_2O or D_2O) for time dependent hydration studies the procedure is identical with the following exceptions. Prior to NMR analysis, NMR tubes were removed from the cold box at -40°C to an ice-salt bath maintained at -3°C and held for 10 minutes. The samples were then transferred to an ice water bath at 0°C for one minute (H_2O) and at 4°C for one minute (D_2O), and finally to the NMR probe maintained at the desired temperature. The sample is held at the probe temperature for 6 minutes prior to the start of data collection.

For preparation of work-free ice-flour mixtures at variable D_2O content (8%-55%), a slightly different procedure was employed. For doughs below 26% moisture the H_2O depleted flour was hydrated in a desiccator over D_2O for varying time periods to achieve the desired moisture content. For D_2O levels above 26%, powdered ice (D_2O) was added to 26% D_2O -hydrated flour as described above. The work-free dough samples were equilibrated at room temperature for a period of at least one hour prior to NMR measurements. For all samples the % moisture was obtained gravimetrically by heating in an oven at 100°C for a period of two days.

NMR Measurements

All measurements were performed at 7.05 tesla on a XL-300 NMR spectrometer (Varian Associates, Palo Alto, CA), ^1H resonance frequency = 300 MHz, ^2H resonance frequency = 45 MHz, equipped with a high power rf amplifier (model 2002A, Henry Radio, Los Angeles, CA) and a variable temperature accessory. The 90° pulse length was maintained at 13 μsec for both ^1H and ^2H NMR studies. The sweep width and number of complex time domain points collected were 40 KHz, 832 points for ^1H and 10 KHz, 1024 points for ^2H . The Carr-Purcell-Meiboom-Gill (CPMG) sequence $[90_x^\circ - (\tau - 180_y^\circ - 2\tau - 180_y^\circ - \tau)_n - \text{half-echo-acquire}]$ was employed for all spin-spin (T_2) measurements [18]. The inversion recovery method ($180^\circ - \tau - 90^\circ - \text{acquire}$) was used for the spin-lattice (T_1) determinations.

For all T_2 measurements the sequence-repetition-period (TR) was $3 \times T_1(^1\text{H})$ and $5 \times T_1(^2\text{H})$. At least four free induction decay (FID) transients or half-echoes were collected at each evolution time for both T_1 and T_2 measurements.

The measurements on fixed concentration doughs (either 35% or 45% moisture) during hydration were carried out at 3° or 30°C (^1H) and 7° and 30°C (^2H). For the freezing curve experiments, the dough was allowed to equilibrate at each temperature for a period of 30 minutes prior to data collection. For experiments monitoring time dependent hydration, T_2 assays were repeated every 6 and 4 minutes for ^1H and ^2H , respectively. For ^1H measurements, fifty even half-echoes were collected (from 0.26 to 114.6 msec) for dough samples prepared at 45% moisture, while 30 even half-echoes were sampled (0.26-60.6 msec) on 35% moisture doughs. In all T_2 measurements (except when noted otherwise) the interpulse spacing (TE) or 2τ time was maintained at 100 μsec . For respective ^2H measurements sixty even-echoes were collected on both 35% and 45% moisture doughs with 2τ time equal to 100 μsec .

The studies varying the 2τ value with a fixed water concentration of work-free doughs (45% moisture) at equilibrium were carried out at 30°C . Approximately 60 even half-echoes were collected at net evolution times ranging from 0.2 to 114 msec in cases where 100 μsec interpulse spacings were employed. In cases where longer 2τ values were used, fewer spin-echoes were sampled. The NMR collection conditions were customized for the ^2H studies of work-free doughs ranging from 8-55% moisture. For samples at low moisture levels only 20 echoes were collected. Intermediate hydration levels required 40 echoes while 60 echoes were employed for the high moisture content samples.

Data Analysis

The time-domain NMR signals (FID's or the latter half of each echo)

were Fourier transformed (with line broadening of 200 Hz and 80 Hz for ^1H and ^2H , respectively), yielding an array of standard absorption mode frequency spectra collected at different net evolution times. The ^1H NMR spectra were dominated by the intense water resonance except at long evolution times when, with the great diminution of the water resonance, there was some evidence of signals arising from fats, protein or carbohydrate (deuterium spectra showed no contamination from these other signals). The water resonance amplitude was quantified by digital integration. Magnetization relaxation for water in the dough systems was, thus, represented by a plot of frequency domain signal integral (I) as a function of net evolution time (t). In order to establish the number of distinct water states defined by T_2 decay, the data were fit to a sum of decaying exponentials each with a different decay time constant, Eq. (1),

$$I(t) = A + \sum_{i=1}^m B_i \exp\left(-\frac{t}{T_{2i}}\right), \quad m = 1, 2 \text{ or } 3. \quad (1)$$

The curve fitting was carried out by use of either RS/1 software (BBN Software, Northbrook, IL) which employs a non-linear least squares algorithm or by use of software developed in-house (vide infra). All magnetization decay profiles were described well by the sum of one, two or three (depending on TE's and hydration level) exponential terms. Two criteria were used to evaluate for, and discriminate between, one, two or three exponential-decay component behavior. The first method, employing a least-squares "goodness of fit" criterion, minimized the variance between the experimental data and the calculated decay curves for each term (i) of the exponential. If the additional exponential term failed to improve the goodness of fit based on a given level of variance criterion, then the additional exponent was designated insignificant. The second criteria employed Bayesian probability theory [19]. This method essentially calculates the model probability of one, two and three exponent fits to the signal decay data. In all cases probability theory greatly favored either one, two or three relaxation components such that assignment of the most probable model was unambiguous. Both probability theory and goodness of fit criteria yielded similar results. One other method was used to analyze the NMR data for multicomponent T_2 relaxation behavior on selected data sets. This method involved fitting the magnitude of the first complex time-domain data point in the (half) spin-echo collected at each net evolution time to the multicomponent exponential decay expression. The magnitude of the first time-domain data point should, in principle, represent the total transverse magnetization of the sample, i.e., the equivalent of integrating the entire frequency spectrum.

RESULTS AND DISCUSSION

Observation of Three Water States

Figures 1 and 2 show ^1H and ^2H T_2 decays (semilog plots) obtained from work-free hard flour dough samples after one hour equilibration using the CPMG method with 100 μsec interpulse spacings and digital integration of the frequency domain absorption spectrum. These transverse relaxation profiles both show strong evidence for three component exponential decay behavior for H_2O or D_2O hydrated samples. Previous reports on mixed flour dough revealed two component magnetization decay [7,8]. This observation defines three water domains in work-free dough that are characterized by different average correlation times (mobilities) and that are in limited communication (i.e., slow exchange) on the time scale presented by this experiment (i.e., 100 μsec). The solid, dashed, and dot-dashed lines in Figs. 1 and 2 represent, respectively, hydration states defined by a relatively long T_2 (23 msec - H_2O , 13 msec - D_2O), an intermediate T_2 (6 msec - H_2O , 5 msec - D_2O) and a short T_2 (0.4 msec - H_2O , D_2O). Work-free soft wheat flour doughs show similar water compartmentalization with slight differences in T_2 times. The fraction of total water represented by each domain (Table 1) is readily derived from the B_i terms in Eq. (1) and is identical (within experimental error) for both ^1H and ^2H CPMG analysis with 100 μsec 2τ times. We assign (label) the three water states in order of increasing T_2 to highly perturbed or immobilized water, intermediate perturbed water and relatively "free" or mobile water. This labeling reflects the normal association of a short T_2 with molecular immobility (long correlation time) while a relatively long T_2 is associated with greater mobility (short correlation time). This labeling scheme is consistent with prior assignment of tightly bound, intermediate bound, and free water. As water is believed to be physically bound to a macromolecule for only a very short time the terminology of "bound water" is probably not appropriate in these systems.

Table 1 includes the results of relaxation analysis carried out on sample preparations at 45% moisture where the magnitude of the first complex time domain point of the half-echo represents total transverse magnetization. This is essentially equivalent to the method commonly used for obtaining T_2 relaxation profiles, i.e., when only a time-domain signal is utilized in the absence of Fourier transformation. Again, as with the frequency-domain resonance area analysis, three water states are defined by three distinct T_2 's for both ^1H and ^2H measurements. This confirming observation of three exponential decay components is not unexpected as the first complex data point of the half-echo is equal to the summed amplitude (total integrated frequency-domain absorption intensity) of all NMR signals present (i.e., water). However, the frequency domain peak area determination provided greater signal-to-noise than did determination of the

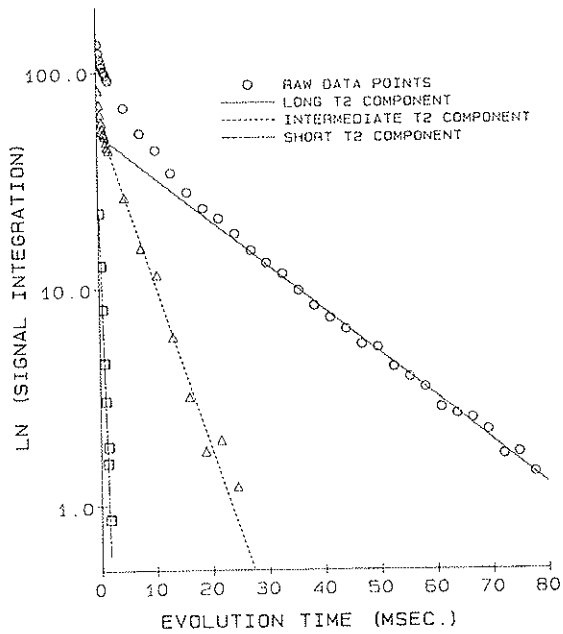


Fig. 1. Semilogarithmic plot of proton transverse magnetization decay generated via a CPMG pulse sequence with $TE=100 \mu\text{sec}$; the sample was work-free hard flour dough of 45% moisture (H_2O) content prepared as described in the main text. The open circles represent the raw data (integrated frequency domain resonance area vs. evolution time) and the solid line through the open circles the best fit to the long T_2 component. The open triangles represent the raw data after subtraction of the long T_2 component and the dashed line through the open triangles the best fit to the intermediate T_2 component. The open squares represent the raw data after subtraction of both the long and intermediate T_2 components. The dot-dashed line through the open squares is the best fit to the short T_2 component.

magnitude of the first point in the half-echo and this led to greater precision (less scatter) in the relaxation decay profile. Parameter estimates taken from integrated resonance area decay curves are, thus, likely to be more accurate.

It is unrealistic to assign a physically unique state of water to each of the three observed T_2 components as it seems reasonable that a complex macromolecular system such as dough would have water present in numerous motionally distinct environments. Water environments are perhaps best characterized in terms of a distribution of adsorption energies (energy-wells) corresponding to a distribution of relaxation times (mobilities) [10]. That only three exponential relaxation decay components are resolved

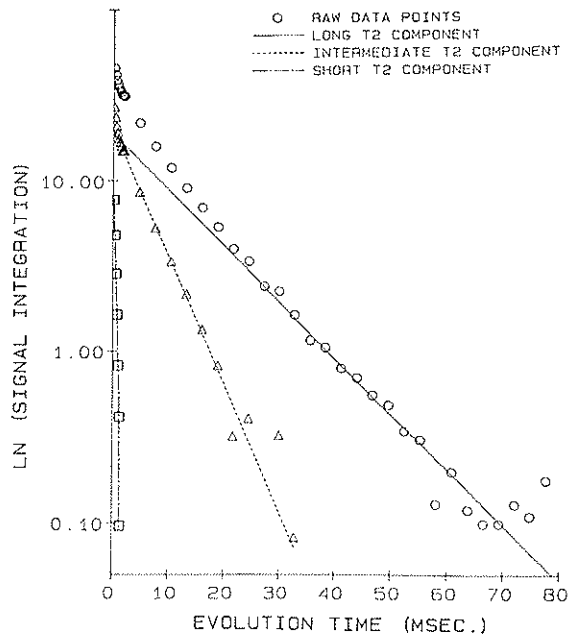


Fig. 2. Semilogarithmic plot of deuteron transverse magnetization decay generated via a CPMG pulse sequence with $TE=100$ μ sec; the sample was work-free hard flour dough at 45% moisture (D_2O) content prepared as described in the main text. Other aspects of Fig. 2 are as described in the caption to Fig. 1.

further suggests many water states possess similar average motional character and/or that rapid chemical/physical exchange is present (rapid with respect to 2τ) and serves to average the expected multitude of hydration motional environments or perturbed water environments into three

Table 1. Occupancy and T_2 times for the three water components observed in 45% moisture work-free hard flour dough by both 1H and 2H NMR measurements. The results from both frequency domain and time-domain first point magnitude methods are presented. The water concentration is expressed in g water/g dry solids (DS).

Analysis Method	Long T_2 Component Fraction (g H_2O /g DS)	Long T_2 (ms)	Medium T_2 Component Fraction (g H_2O /g DS)	Medium T_2 (ms)	Short T_2 Component Fraction (g H_2O /g DS)	Short T_2 (ms)	N^* (trials)	
Integration of frequency spectrum	1H	0.28	20.6	0.31	6.0	0.23	0.4	5
	2H	0.31	12.7	0.29	5.6	0.22	0.4	5
First complex point of FID	1H	0.25	20.6	0.32	5.7	0.25	0.4	2
	2H	0.30	12.0	0.21	3.5	0.31	0.4	2

T_2 -distinguishable states (vide infra). Another way to state this is that water samples many different environments rapidly compared to its relaxation rate and that what is observed is the average relaxation rate of all environments [10]. In this configuration, the highly perturbed water component represents a distribution of molecules with restricted motion (corresponding to deep wells on a potential energy surface) which are exchanged with one another at a rate in excess of the $1/T_2$ between each individual environment [20]. The water domain of intermediate mobility may represent a similar distribution of environments in which water is less affected by protein and carbohydrate macromolecules from the flour. The water compartment represented by an intermediate T_2 value may also correspond to entrapped water that is contained within starch granules and thus may have intermediate order. The compartment with the longest T_2 is "freezable" and is, thus, consistent with a free water phase (Fig. 3, Table 2). Lillford and coworkers propose a model which incorporates a distribution of probabilities of water exchanges as an explanation for multicompartiment relaxation behavior [17]. In these models, multi-exponential relaxation decay is associated with sample heterogeneity. Alternatively, cross relaxation processes and not water mobility could account in part for the observed three exponential relaxation decay of water in work-free doughs [21,22]. The fact that ^1H and ^2H data are similar, i.e., both show 3 components with roughly the same fractional compartmentalization, argues against this explanation as ^2H should be affected little by cross relaxation. The T_2 relaxation experiment "operationally" defines water as constrained to only three states of distinct "average" mobility which are apparently not in rapid chemical/physical exchange or such relaxation discrimination would not be possible.

The interpretation of NMR data regarding water mobility in complex macromolecular systems is largely model dependent.[2,3] The fast exchange model would attribute the observed T_2 times to weighted average of T_2 values for water states in rapid exchange [9,10]. Therefore, the T_2 measured for "free water" in dough represents the mole fraction weighted average T_2 from bulk water and some fraction of perturbed water (that is in rapid exchange with the bulk water) and is thus, considerably shorter than that of bulk water. The T_2 temperature dependence behavior for the long T_2 component, vide infra, suggests this component is in intermediate exchange. Since water adsorption by macromolecules is largely reversible it seems reasonable to postulate that all NMR-derived relaxation rates for water in dough likely result from average motional states modulated in part by chemical exchange with bulk water. This may be true even for the shortest T_2 observed, although it seems more reasonable to postulate that the highly immobilized fraction represents an averaging effect of "bound" water sites of varying T_2 's

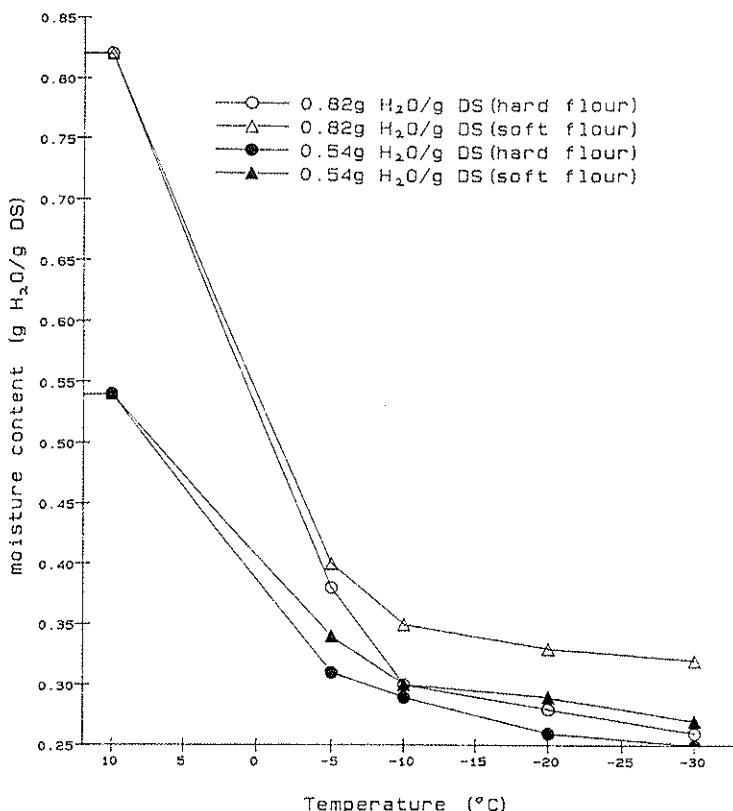


Fig. 3. Variable temperature curves as determined by ^1H transverse relaxation measurements of work-free hard and soft wheat flour doughs. The samples are prepared at 35% and 45% moisture corresponding to 0.54 and 0.82 g $\text{H}_2\text{O}/\text{g}$ dry solid, respectively. NMR measurements were carried out by the CPMG method employing $\text{TE}=100$ usec. The sample was equilibrated at each temperature for 30 minutes prior to data collection. The open and filled circles correspond to NMR observable water for the 45% and 35% moisture hard flour dough samples, respectively. The open and filled triangles correspond to 45% and 35% moisture soft flour dough samples, respectively.

in exchange with each other. Alternative models would predict water correlation times to be orders of magnitude shorter than predicted by an exchange model due to cross relaxation or to a hydrodynamic effect [22,23].

In "as is" hard wheat flour (ca. 14% moisture) a single water T_2 of 0.7 msec is observed. This broad (~ 500 Hz $\Delta \nu_{1/2}$) water resonance is not observed in flour dried under vacuum to ca. 1% H_2O . One expects water to be largely associated with protein or carbohydrate in "as is" flour and the short T_2 value observed is consistent with this, i.e., the T_2 is consistent with greatest immobility. It is somewhat curious, however, that an even shorter T_2 of 0.4 msec is observed for the highly perturbed water component when the flour has been hydrated to work-free dough at 45% moisture. One

Table 2. Variable temperature studies of water components in work-free hard and soft flour doughs as measured by ^1H relaxation methods. The dough samples are equilibrated at each temperature for 30 minutes prior to relaxation measurements. The moisture content, given on a dry solids basis, is established by extrapolating the water components (1, 2 or 3) to zero evolution time.

45% moisture (0.82 g $\text{H}_2\text{O/g}$ DS)

Temp. ($^{\circ}\text{C}$)	Hard Flour Component fractions (g $\text{H}_2\text{O/g}$ DS)	T_2 (ms)	Soft Flour Component fractions (g $\text{H}_2\text{O/g}$ DS)	T_2 (ms)
30	0.29	21.4	0.35	22.0
	0.34	7.0	0.27	5.9
	0.19	0.43	0.20	0.38
10	0.26	22.1	0.27	24.8
	0.33	6.8	0.33	6.5
	0.23	0.40	0.22	0.39
-5	0.26	2.5	0.27	2.3
	0.12	0.40	0.13	0.31
-10	0.19	1.6	0.24	1.6
	0.11	0.36	0.11	0.34
-20	0.17	1.0	0.20	1.1
	0.11	0.3	0.13	0.33
-30	0.26	0.8	0.19	0.7
			0.13	0.23

35% moisture (0.54 g $\text{H}_2\text{O/g}$ DS)

Temp. ($^{\circ}\text{C}$)	Hard Flour Component fractions (g $\text{H}_2\text{O/g}$ DS)	T_2 (ms)	Soft Flour Component fractions (g $\text{H}_2\text{O/g}$ DS)	T_2 (ms)
30	0.10	15.6	0.13	13.9
	0.30	5.3	0.26	4.1
	0.14	0.36	0.15	0.33
10	0.09	17.4	0.13	14.5
	0.30	5.3	0.27	3.9
	0.15	0.38	0.14	0.37
-5	0.19	1.74	0.21	1.53
	0.12	0.41	0.13	0.48
-10	0.18	1.56	0.30	1.19
	0.11	0.42		
-20	0.15	0.93	0.29	0.75
	0.11	0.28		
-30	0.15	0.79	0.27	0.48
	0.10	0.25		

would anticipate observing a somewhat longer T_2 than that found in the natural flour for this water component in dough, due to predicted exchange with less immobilized water. That this is not seen may simply reflect the substantial experimental inaccuracy in determining this short component, i.e., 0.4 ms and 0.7 ms T_2 's may be equivalent within the error of the relaxation analysis. However, there also may be structural changes in flour gluten or starch at 45% moisture content which account for this apparent discrepancy. Furthermore, the assumption of a direct T_2 -mobility

correlation is based on isotropic water motion. This may be an oversimplification in the dough system. Effects of cross relaxation which are reported for hydrated proteins also may contribute to the ^1H T_2 differences observed for the immobile water component in "as is" flour and 45% moisture doughs [12,23].

The agreement between ^1H and ^2H NMR measured water component fractions is excellent (Tables 1 and 2), although the primary relaxation mechanism for each nuclide is different, i.e., through space nuclear dipole-dipole interaction for ^1H and through nuclear quadrupole interaction with local electric field gradients for ^2H . For the ^1H data both cross relaxation between water and other nearby protons and signal "contamination" due to contributions from mobile protons in protein, carbohydrate, and fats may occur in principle. (Rigid protons in proteins and carbohydrates should exhibit T_2 's < 200 μsec , and negligible signal contribution is expected from these species at longer evolution times.) However, based on the ^2H data for which such considerations are expected to be slight, these effects do not appear to alter the observed fractional compositions of water states as defined by ^1H NMR. Differences in ^1H and ^2H T_2 's (Table 1) can be rationalized by noting that the more efficient quadrupolar relaxation pathway is available only for the deuteron.

Freezing Studies

Table 2 lists results ($\% \text{H}_2\text{O}$, T_2 times) of ^1H NMR freezing studies carried out on work-free hard and soft flour doughs. Above 0°C , three motionally distinct water compartments are readily separated in both flour types. Upon cooling to -5°C , however, the long T_2 compartment disappears, i.e., at -5°C , the transverse relaxation decay is made up of two rather than three exponential terms. Presumably the fraction of water which gave rise to the long T_2 component becomes NMR invisible, under our collection conditions, because at -5°C its mobility is greatly restricted (solid ice). Curiously, the amount of water signal lost in going to -5°C is greater than the long T_2 component above 0°C suggesting some fraction of the highly and intermediately perturbed water is freezable as well. A reasonable explanation for this observation is that the free water fraction is in exchange with water in the fractions characterized by shorter T_2 times. As the free water freezes at -5°C , any water diffusing from a hydration site (on protein or carbohydrate) also becomes freezable, but no freely diffusible free water is then available to rehydrate the vacant hydration site.

Cooling the doughs to temperatures below -5°C causes further loss of NMR observable water. The water loss occurs predominately from the intermediate T_2 compartment. In some cases (soft flour 35% moisture at -10° and below and hard flour 45% moisture at -30°C) only one water component is

observed by NMR. In these cases the T_2 time is commonly intermediate between the two shorter T_2 fractions characterized at higher temperatures. We expect this results from a contribution of intermediate water component being present but non-resolvable by our transverse relaxation analysis. This is a common problem encountered in deconvolution of multiexponential decays, i.e., when one component is present to only a small extent or when decay time constants are similar.

The NMR-observable signal decreases further as the temperature is lowered to -30°C . This observation is typical for freezing curves of many food systems studied by NMR methods [24]. This NMR observation is consistent with a loss of motion in the amorphous non-freezable water component. For our freezing studies we held the sample at each temperature for 30 minutes prior to analysis by NMR. We find the dough sample to be at a "pseudo-equilibrium" under these conditions in that holding them for a period of several hours results in no apparent change in the observable water. It is possible that all of the non-freezable water we observe at, say, -10°C would freeze if the sample was held below its freezing point for an infinitely long period.

Figure 3 shows profiles of water (% of total sample) as a function of temperature for work-free doughs. Note the freezing curves show clear differences between hard and soft flour hydration, especially at lower temperatures. Specifically, the amount of NMR observable signal is greater for soft than hard flours for the same total water content. This result suggests water in soft flour dough has greater mobility than that in hard-flour. This may be the result of the greater protein content, commonly associated with hard flours (the hard wheat flour has ~12% protein compared to ~6% for soft wheat flour). Flour proteins (glutens) are known to have high water adsorption capacity and may account in part for the differences in non-freezable water fraction. Differential thermal analysis studies of hard and soft flour doughs yield contradicting evidence for this, however, as the high energy binding states (associated with NMR invisibility) are felt to be associated with starch rather than protein [25].

Effect of Long Echo Time (TE)

In pulsed NMR, T_2 measurements are carried out by sampling refocused transverse magnetization (spin-echoes). Refocusing methods eliminate signal decay caused by static magnetic field inhomogeneities within the sample and associated deleterious effects from molecular diffusion [26,27]. Additionally, chemical/physical exchange effects which limit the average lifetime of a spin configuration are reduced via spin echo methods carried out at short TE times [28]. As defined here, the 2τ time (TE) or, alternatively, the interpulse spacing, is the time between successive echos. Pulse sequences

which take advantage of refocused magnetization such as the CPMG method allow one to approach the natural T_2 decay which, in the absence of the effects mentioned above, reflects the mobility of a nucleus (molecule). Choice of the 2τ employed in a multipulse experiment is especially important in cases where the molecule of interest is in exchange with one or more motionally unique environment(s). For example, consider water which is in exchange between a bound configuration (motional environment A) and free bulk water (motional environment B) with equivalent chemical shifts. If 2τ is chosen to be significantly shorter than the lifetime and T_2 of water in each environment, the observed transverse magnetization will decay biexponentially. Analysis of the decay profile [see Eq. (1)] yields characteristic relaxation times (T_{2A} , T_{2B}) and the fraction of water in each environment (via B_A , B_B). If instead 2τ is chosen significantly longer than the lifetime of water in each environment, the observed transverse magnetization decays with a profile defined by a single exponential time constant which represents the mole fraction weighted average of both water environments A and B (Eq. (2)).

$$\frac{1}{T_{2, \text{observed}}} = \frac{X_A}{T_{2A}} + \frac{X_B}{T_{2B}} \quad (2)$$

More generally, for n motionally distinct environments for water all in rapid exchange relative to TE (all site lifetimes short relative to TE), the observed single exponential time constant is given by Eq. (3),

$$\frac{1}{T_{2, \text{observed}}} = \sum_{i=1}^n \frac{X_i}{T_{2i}} \quad (3)$$

where X_i and T_{2i} represent, respectively, the mole fraction and spin-spin relaxation time of spins in site i .

Table 3 and Fig. 4 show results of T_2 measurements on dough as a function of 2τ . Varying 2τ causes a dramatic effect on (a) the number of water states defined by the relaxation experiment, (b) the apparent fractional composition of water states and (c) the T_2 associated with each state. These results suggest one must exercise caution in assigning water state compartmentalization by NMR methods, especially when making comparisons to results from other physical methods, as the results obtain depend critically on the 2τ chosen.

The loss of the tightly bound water component with increasing 2τ is readily explained. In considering the CPMG sequence, the shortest 2τ used (100 μsec) makes data acquisition possible at even-echo evolution-times of 200, 400, 600, ..., μsec and, thus, provides sufficient though certainly not optimal sampling of the short, immobilized, component (e.g., $3 \times T_2 = 1.2$ msec for 95% decay during which 6 evolution times are sampled at 2, 4, 6, 8,

Table 3. Water compartmentalization in 45% moisture work-free hard flour dough measured as a function of the echo time (2τ) in the CPMG sequence. The standard deviation for parameter estimates is given in parentheses for cases where multiple samples were evaluated at the same echo time, 2τ .

Proton (H_2O) Relaxation							
TE (2τ)	Long T_2 Component Fraction (g H_2O /g DS)	Long T_2 (ms)	Medium T_2 Component Fraction (g H_2O /g DS)	Medium T_2 (ms)	Short T_2 Component Fraction (g H_2O /g DS)	Short T_2 (ms)	N ^a (trials)
100 μ sec	0.28(0.01)	20.6(1.6)	0.31(0.02)	6.0(0.9)	0.23(0.01)	0.41(0.10)	5
300 μ sec	0.34	20.9	0.48	5.0			1
600 μ sec	0.52	14.9	0.30	6.5			1
900 μ sec	0.57(0.04)	11.1(1.1)	0.25(0.04)	3.1(0.6)			5
1200 μ sec	0.52	18.9	0.30	5.6			1
1600 μ sec	0.32	17.1	0.50	5.8			5
2 msec	0.20(0.05)	16.7(0.5)	0.62(0.05)	6.0(0.02)			5
4 msec		8.3(0.3)					5

Deuteron (D_2O) Relaxation							
TE (2τ)	Long T_2 Component Fraction (g H_2O /g DS)	Long T_2 (ms)	Medium T_2 Component Fraction (g H_2O /g DS)	Medium T_2 (ms)	Short T_2 Component Fraction (g H_2O /g DS)	Short T_2 (ms)	N ^a (trials)
100 μ sec	0.31(0.03)	12.7(0.7)	0.29(0.3)	5.0(0.8)	0.22(0.01)	0.40(0.06)	5
900 μ sec	0.56(0.06)	9.2(0.6)	0.26(0.06)	2.0(1.2)			5
2 msec	0.25(0.06)	14.2(1.6)	0.57(0.06)	6.2(0.5)			5
4 msec		9.6(0.5)					5

^aStandard deviations for parameter estimates are given in parentheses for cases where multiple 0.1 cm samples were evaluated using the same echo time, 2τ .

10 and $12 \times 2\tau$). The next longest TE used (300 μ sec) allows data sampling at even-echo evolution-times of only 600, 900, 1200, ... μ sec and, thus, largely misses sampling the rapidly decaying magnetization from the immobilized water (only 20% of the magnetization remains at the first even echo). Prior NMR reports of water in mixed dough systems found only two components likely due to choice of a long interpulse spacing value [7,8]. In characterizing water, one must choose a 2τ value which is not too short, as the echos will contain substantial contributions from protein magnetization. In using short 2τ times of less than ca. 300 μ sec, there will also be some degree of spin-locking on the water magnetization. At an interpulse spacing of 100 μ sec, spin locking effects should be significant. This will result in apparent T_2 times greater than the natural T_2 's for the water compartments in flour doughs but should not affect our ability to distinguish water components.

The derived fractional compositions of water states show an unusual trend with increasing 2τ value (Table 3, Fig. 4). The changes observed in going from 100 to 300 μ sec TE are expected, as there is a difference in the number of components observed ($3 \rightarrow 2$, vide supra). The apparent increase of free water with increasing TE and flip-flop in water domain occupancy fraction above 1.2 msec TE is less easily explained. This trend is observed for both 1H and 2H studies, suggesting that contributions from cross relaxation are unlikely. Possible explanations for observed (relaxation

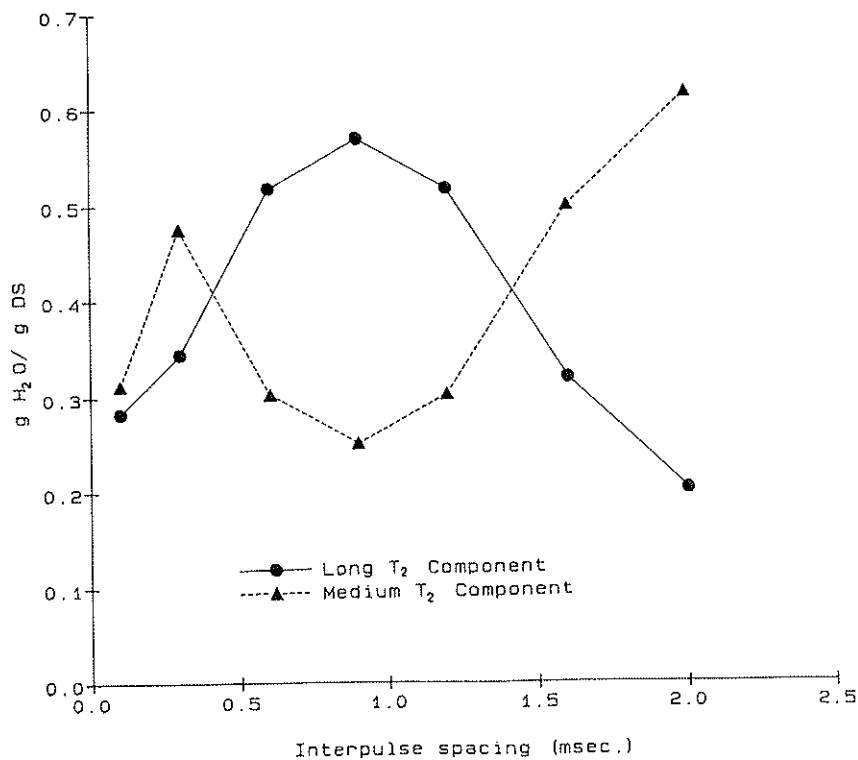


Fig. 4. Dependence of water component occupancy on the CPMG interpulse spacing value. The transverse magnetization decay in hard flour dough, prepared at 45% moisture, is measured and analysed as the 2τ time is increased. The filled circles and triangles represent the water occupancy [dry solids (DS) basis] vs. 2τ as defined by the NMR method. Only the two (out of three observed) water components are represented at the $2\tau=100$ μ sec data point. The longer 2τ 's chosen reveal only two components except at 4 msec when single exponential decay is observed.

measurement defined) increased occupancy of the free water fraction include decreased effects of spin-locking [29] and increased effects of water exchange [28] with increasing interpulse spacing. The corresponding decrease of T_2 times over the same regime ($2\tau = 100$ to 900 μ sec) is also consistent with water exchange rates being on the same time scale as the 2τ , i.e., water in intermediate exchange on the transverse magnetization refocusing time scale [5]. The apparent flip-flop of water state occupancy fractions at a 2τ above 1.2 msec may reflect both exchange effects and inadequate sampling of the intermediate decay component. At a 4 msec interpulse spacing, only a single exponential decay, consistent with only one relaxation defined water state, is observed with T_2 intermediate between that found for free and intermediate perturbed water. This suggests these two water domains are in rapid exchange on a time scale of 2τ .

Hydration Studies

NMR relaxation studies of the hydration dynamics of work-free doughs (35% and 45% moisture) were carried out at both 30° and 3°C (7°C for ^2H studies). Figure 5 shows representative time courses of the NMR defined water compartments. Table 4 includes water occupancy and T_2 times for 50 and 64 minute post-thawing ^1H and ^2H studies, respectively (hydration "equilibrium" was considered to be complete about one hour post-thawing). The reproducible feature in these studies is a loss of water from the free fraction (longest T_2 component) and a buildup of the medium T_2 water fraction, as shown in Fig. 5. The water associated with the shorter T_2 compartment also increases slightly during the same time course. The ^2H data yielded more consistent results in this experiment than the ^1H studies (less scatter in repetitive trials) but

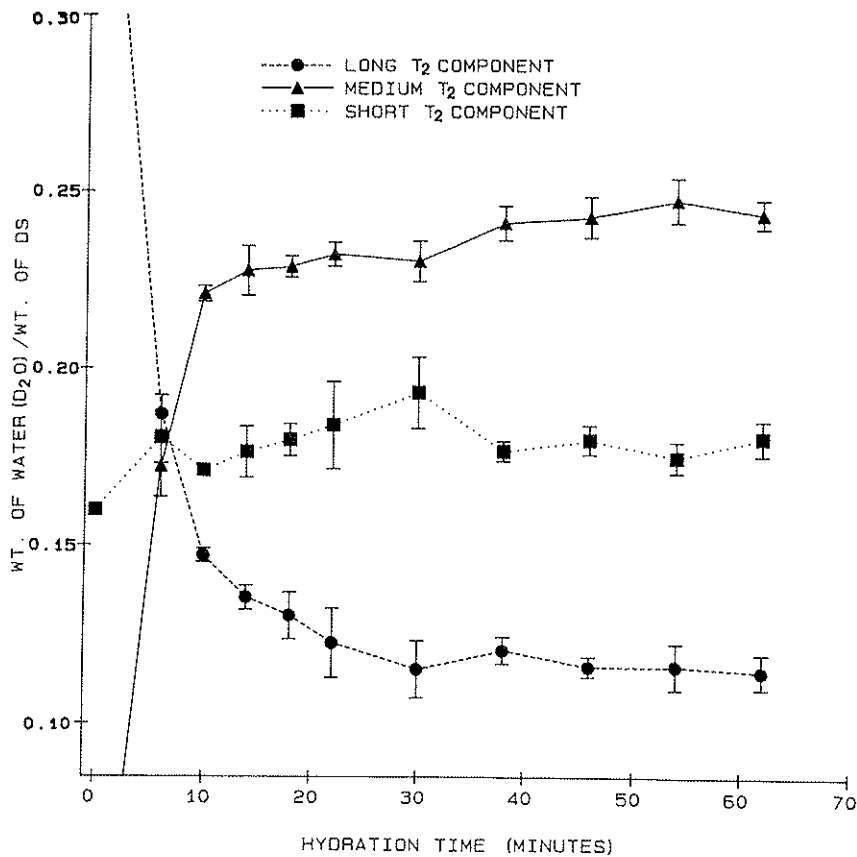


Fig. 5. Hydration profile for 35% D_2O work-free hard flour dough at 7°C.

Wheat flour-water (D_2O) mixtures are prepared at -40°C and allowed to thaw above the freezing point. The transverse magnetization decay is measured during the equilibration period and water occupancy for the three components is determined. The triangles, squares and circles represent water occupancy for the medium, short and long T_2 component, respectively. The vertical bar on each symbol represents one standard deviation based on five separate trials.

Table 4. Equilibrium (one hour post-thaw) water compartment analysis [dry solids (DS) basis] based on ^1H and ^2H transverse relaxation decay for work-free soft and hard flour doughs. The ^1H and ^2H data has a relative standard deviation of 15% and 7%, respectively, based on five trials for each measurement.

Nuclide	Flour Type	Temperature of Hydration (°C)	Long T_2 * Component Fraction $\frac{\text{g H}_2\text{O(D}_2\text{O)}}{\text{g DS}}$	Long T_2 (ms)	Medium T_2 Component Fraction $\frac{\text{g H}_2\text{O(D}_2\text{O)}}{\text{g DS}}$	Medium T_2 (ms)	Short T_2 Component Fraction $\frac{\text{g H}_2\text{O(D}_2\text{O)}}{\text{g DS}}$	Short T_2 (ms)
45% moisture (0.82 g Water/g DS)								
^1H	SOFT	3°	0.34	30.2	0.28	5.4	0.20	0.34
^2H	SOFT	7°	0.33	23.0	0.24	3.4	0.25	0.44
^1H	SOFT	30°	0.27	27.0	0.26	5.6	0.30	0.37
^2H	SOFT	30°	0.31	16.7	0.27	3.7	0.24	0.37
^1H	HARD	3°	0.26	22.1	0.27	5.8	0.29	0.39
^2H	HARD	7°	0.27	24.7	0.30	4.4	0.25	0.51
^1H	HARD	30°	0.28	20.5	0.25	6.3	0.30	0.40
^2H	HARD	30°	0.28	16.0	0.29	4.4	0.23	0.45
35% moisture (0.54 g Water/g DS)								
^1H	SOFT	3°	0.15	15.1	0.22	3.8	0.17	0.38
^2H	SOFT	7°	0.10	14.5	0.25	2.5	0.20	0.51
^1H	SOFT	30°	0.12	13.5	0.24	4.2	0.17	0.32
^2H	SOFT	30°	0.17	11.1	0.20	2.8	0.17	0.38
^1H	HARD	3°	0.16	12.9	0.20	4.0	0.18	0.37
^2H	HARD	7°	0.11	15.0	0.24	2.8	0.18	0.48
^1H	HARD	30°	0.08	16.6	0.26	5.0	0.19	0.36
^2H	HARD	30°	0.14	11.5	0.25	3.2	0.14	0.39

* ^1H water fractions have a relative standard deviation of about 15%.

^2H water fractions have a relative standard deviation of about 7%.

both methods show similar trends. The most notable feature is the very rapid uptake of free water (from the melted ice) by the dough. The time scale of our T_2 measurement is too long to accurately quantitate the three water compartments in the region where hydration is most rapid (less than 5 minutes after thawing). Because of this we are unable to make any distinction between the rate of hard and soft flour hydration. Based on the more gradual changes occurring after the first five minutes post-thawing, we can conclude that hydration in the absence of work appears to be complete after 40 minutes. Additionally, we have found no evidence for continued changes in the NMR-defined hydration compartments at times up to 3 hours post-thawing. The "equilibrium" water distribution shown in Table 4 reveals a substantial difference in occupancy of the free water compartment between doughs prepared at 35% and 45% moisture. This difference presumably reflects substantial saturation of the two shorter T_2 fractions at 45% moisture and, thus, accounts for the larger reservoir of mobile water. At the 35% moisture level both hard and soft flour doughs show less occupancy of the immobile water fractions by about 23% ($\text{H}_2\text{O/DS}$) compared to 45% moisture samples. The water component identified by the shortest T_2 also shows an increase in population

during hydration. If this component correlates with the short T_2 environment in "as is" flour, then it also reflects considerable hydration in the first 5 minutes post-thawing. For example, for 45% moisture doughs, this fraction reaches a value of ~50% greater, and for 35% moisture, a value of ~10% greater than the starting flour.

The NMR water relaxation measurements reveal only slight differences in hydration characteristics as a function of temperature or flour type. This observation is consistent with previous reports which failed to link NMR observables with baking properties [7,8]. In comparing samples prepared at different moisture levels, an increase in occupancy for all water components is observed at higher water content as expected. However, the increase in free water is striking (~100%), compared to domains characterized by shorter T_2 times. This observation is consistent with flour having a finite capacity for water absorption. The long T_2 component serves essentially as an overflow reservoir for water which cannot be accommodated elsewhere. At 45% moisture, the soft flour doughs on average have 10% more water in the free and less in the medium and tightly "bound" compartments than the hard flour doughs. This trend is consistent with the greater water absorption found in hard wheat flours. Additionally, the free water T_2 shows a reproducible decrease with decreasing moisture content (the medium T_2 shows this to some extent also) and increasing temperature. Both of these observations are consistent with the exchange models [5,9]. From Eq. (2) the observed T_2 can be seen to increase with an increase in long T_2 component (bulk water) if exchange is involved.

Water (D_2O) Titration Experiments

Hard and soft work-free flours were hydrated with D_2O over a range of 8-55% moisture (based on D_2O) and characterized by 2H NMR relaxation methods. Figure 6 shows the T_1 dependence, while Figures 7 and 8 depict the T_2 relaxation results. The T_1 profile appears identical for both flour types while the T_2 analysis reveals a slight increase in the occupancy of the long T_2 water compartment for the soft compared to hard flour doughs. The results from the titration experiment show that water occupies the NMR defined hydration states preferentially, i.e., the bound compartment fills before the medium compartment, etc.

The spin-lattice relaxation vs. moisture concentration curve (Fig. 6) shows a break around 0.2 g D_2O /g dry solid (DS). The T_1 profile can be approximated by two linear regions from 0.1-0.2 and from 0.2-1.2 g D_2O /g DS. The D_2O flour concentration of 0.2 g/DS is very close to the value at which the intermediate T_2 component begins to show occupancy (Figs. 7 and 8) and perhaps signifies a marked change in average water mobility. Below 0.2 g/DS moisture the NMR decay indicates that only the highest energy (lowest

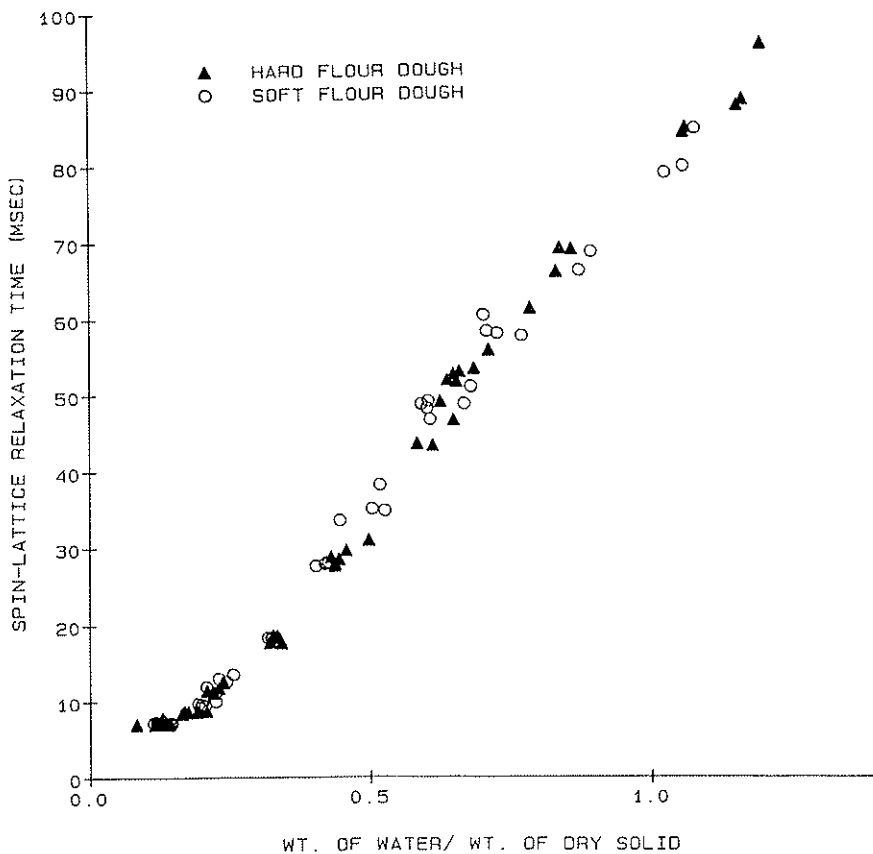


Fig. 6. The ^2H dependence of hard and soft wheat flour doughs as a function of water content. Variable moisture content deuterium exchanged wheat flour is hydrated for a period of at least one hour prior to ^2H NMR T_1 measurements (inversion recovery method) at 30°C . The triangles (\blacktriangle) and circles (\circ) correspond to hard and soft flour preparations, respectively.

mobility) sites are occupied. These are defined by a T_2 time of 0.2-0.4 msec. In fact, even at the highest moisture samples studied, the tightly bound component is characterized by the same T_2 time. In contrast, the T_2 values for the medium and long T_2 components increase with increasing moisture content (Fig. 9). It seems reasonable to correlate the suggested break in the T_1 profile to the onset of hydration-site occupancy by water with much greater mobility than that of the shortest T_2 or highly immobilized compartment. The increase in T_1 and T_2 times shown for both the medium and "free" water components with increased moisture is largely consistent with the predictions of exchange with bulk water, i.e., as the mole fraction of bulk water is increased (long T_2) the observed T_2 's become greater. That the shortest T_2 compartment does not show this trend may indicate that it is in limited exchange with bulk water.

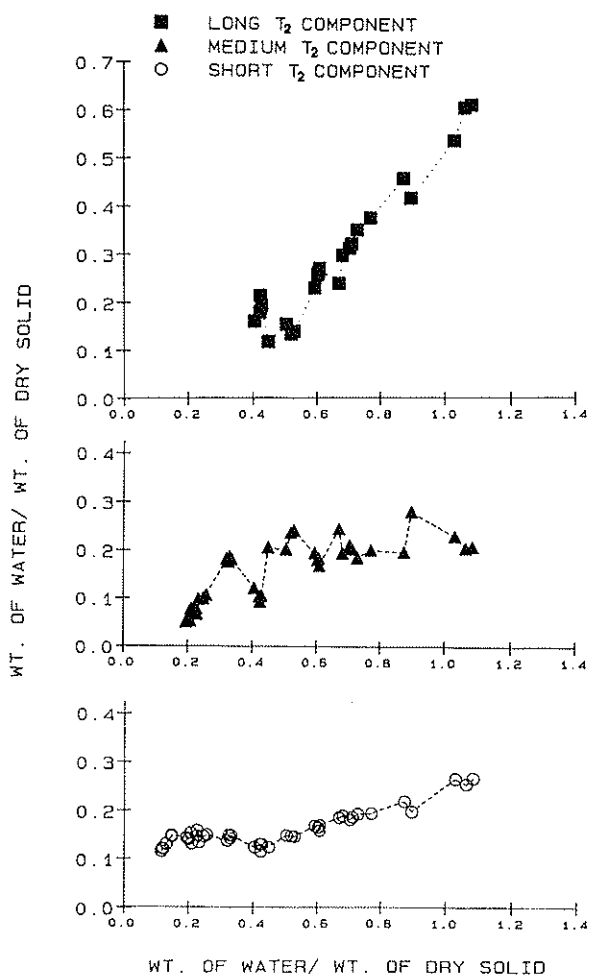


Fig. 7. D_2O titration of hydration sites in work-free hard wheat flour dough. The circles (○), triangles (▲), and squares (■) represent moisture content for the respective short, medium and long T_2 water compartments measured by 2H NMR at $30^\circ C$.

The occupancy of water compartments as determined by transverse relaxation decay shows an initial rapid increase followed by a decrease and a final increase (Figs. 7, 8). The initial rapid occupancy is reasonable, as one expects an abundance of vacant sites on the dry or partially hydrated flour substrate. The decrease is likely an artifact of our non-linear least squares analysis. It becomes difficult to obtain clean separation of multi-exponential decays when either one component is in great excess or the decay constants are similar. At 0.2 g D_2O/g DS, for example, the short T_2 is about 0.3 msec compared to the estimated medium T_2 fraction of about 0.8 msec. As more hydration occurs (below the onset of the "free" water compartment), the medium T_2 increases to ~2.5 msec which enables a cleaner

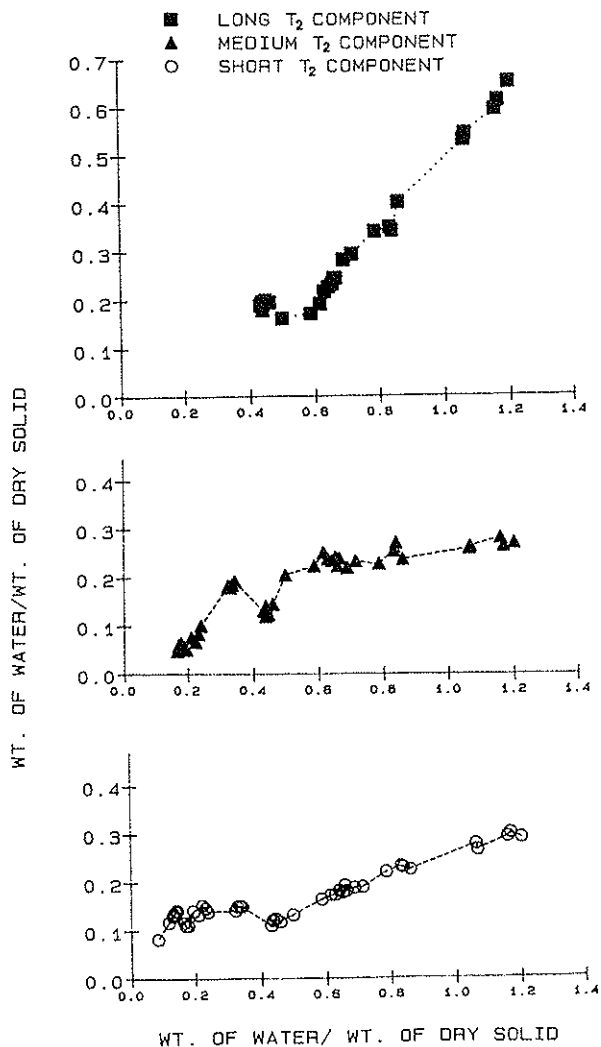


Fig. 8. D_2O titration of hydration sites in work-free soft wheat flour dough. The circles (○), triangles (▲), and squares (■) represent moisture content for the respective short, medium and long T_2 water compartments measured by 2H NMR at $30^\circ C$.

separation between decaying components. At hydration levels of $0.4 \text{ g } D_2O/\text{g DS}$, when the "free" water becomes observable, a similar discrimination problem is seen affecting both the tightly and medium bound sites.

Over the moisture range we investigated, we did not see clear evidence for saturation of any water compartment. The increase in occupancy of the two shorter T_2 compartments clearly slowed from its initial rapid growth, but on average was increasing slightly even at the higher concentration. The "free" water component increases linearly at a rapid rate above $0.5 \text{ g } D_2O/\text{g DS}$, suggesting few remaining hydration sites characterized by short T_2 time.

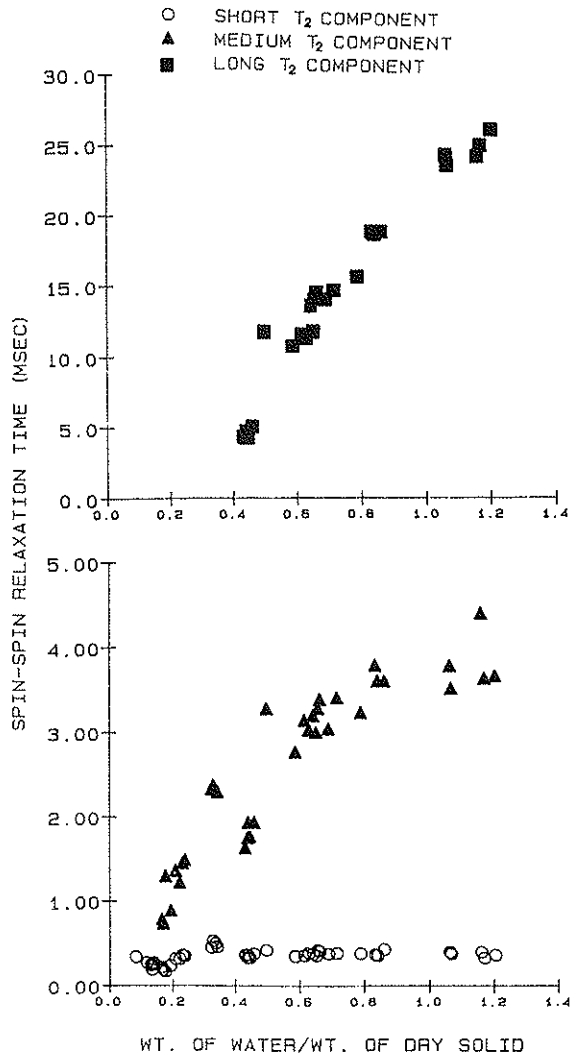


Fig. 9. T_2 time as a function of hydration level.

This T_2 study showed little difference between flour types, except slightly greater water occupancy of the "free" water compartment for soft flour doughs. This same trend was noted earlier in studies of doughs prepared at 35% and 45% moisture content.

CONCLUSIONS

The NMR studies reported here show that work-free flour is characterized by three water compartments, instead of by two as was reported previously on mixed doughs. Additionally, the water compartmentalization is found to depend strongly, and in an unexplained fashion, on the interpulse spacing (2τ) employed.

The NMR studies do show slight differences between hard and soft flour types. These differences include: a) a lower amount of NMR-visible non-freezable water in hard flour doughs; b) slightly longer T_2 times for the two longer T_2 compartments in soft flour; and c) a slightly greater occupancy of the free water component in soft flour dough, especially at high moisture content. The NMR observations may simply reflect the different protein and/or carbohydrate contents of the flours and their relative ability to retain water. It is not clear, however, that product quality, from a baking perspective, can be linked to protein or carbohydrate content of a flour type.

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