Temperature-Induced Changes in the Dynamic Rheological Behavior and Size Distribution of Polymeric Proteins for Glutens from Wheat Near-Isogenic Lines Differing in HMW Glutenin Subunit Composition

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ABSTRACT

Viscoelasticity of hydrated gluten depends on composition of HMW gluten subunits (GS), size distribution of glutenin polymers, and protein-protein interactions. Glutens extracted from four near-isogenic lines with differing HMW-GS were analyzed. Rheological properties were studied by dynamic assay in shear. Size distribution of prolamins was determined by sequential extraction and size-exclusion HPLC. Assays performed at 20°C confirmed that viscoelasticity was determined by large glutenin polymers. The abundance of large glutenin polymers depended on the HMW-GS composition of the lines. Difference of functionality linked to subunit structure was highlighted by comparing the behaviors of the *1A/1B* null and *1A/1D* null lines. Glutens were submitted to heating and cooling cycles, with or without an SH-blocking agent (N-ethylmaleimide [NEMI]). At 20–40°C, no irreversible changes of the mechanical properties occurred. Thermal treatment affected chain mobility, and possibly H bonds, but not the chemical structure of the network. At >40°C, irreversible rheological changes were observed without NEMI. Irreversibility was mainly due to chemical modifications affecting the polymer size distribution through SH-SS exchange reactions. The sensitivity of gluten to temperature depended on subunit composition.

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Allelic variations of the composition of HMW glutenin subunits (GS) of wheat result in large differences in breadmaking properties (Payne et al 1987a). Studies of near-isogenic lines of wheats differing only in HMW-GS composition demonstrated that this effect was due to both the relative amount of HMW-GS in the glutenin polymers and to structural features of the subunits (Payne et al 1987b, Lawrence et al 1988, Popineau et al 1994a). In this respect, *1D*-encoded subunits 5+10 induced better baking performances than subunits 2+12 (Payne et al 1987b; Popineau et al 1994a; Gupta et al 1994a,b). In the same way, in double null lines, 5+10 subunits induced higher gluten viscoelasticity than *1B*-encoded subunits 17+18 (Gupta et al 1995, Hargreaves et al 1996). These variations of subunit composition influence the size distribution of glutenin polymers and their aggregation and extractability. Rheological analyses of gluten and gluten fractions showed a strong correlation between viscoelasticity, or network connectivity, and the amount of the largest glutenin polymers (Cornec et al 1994, Popineau et al 1994a; Gupta et al 1995; Tsiami et al 1997a,b). Furthermore, spectroscopic analyses using Fourier transform infrared on an attenuated total reflectance accessory (FT-IR ATR) indicated that hydrated viscoelastic gluten and gluten subfractions had a higher amount of β-sheet structures than in dilute acetic acid solution, corresponding to Gln- and Pro-rich repetitive domains (Pézolet et al 1992). Proportion of β-sheet structures was related to the amount of large glutenin polymers and thus to network connectivity (Lefebvre et al 1994a, Popineau et al 1994b). Such a relationship was also observed with the mobility of polypeptide chains when examined by electron spin resonance (ESR) spectroscopy (Hargreaves et al 1996). All these results suggested that glutenin polymers could interact in viscoelastic gluten through β-sheet structures. The repetitive domains of subunits, in particular those of the HMW-GS which are very long, could act as junction zones in the network because numerous, periodically distributed Gln residues favor interchain hydrogen bonds (Pézolet et al 1992, Popineau et al 1994b, Belton et al 1994).

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Characterizing the effects of heat treatment on gluten properties is of interest because most of the uses of wheat flours and gluten involve heating or cooking processes. It is also a way to gain a better insight into the organization of gluten network and into the interactions that govern it. Temperature is likely to affect the state of covalent and noncovalent links in glutenin polymers and aggregates. Heat disrupts hydrogen bonds and electrostatic interactions but enhances hydrophobic interactions and accelerates molecular motion. Simultaneously, SH-SS interchanges are facilitated (Schofield et al 1983), giving rise to more aggregation. When no chemical or structural changes alter the system, cooling and heating can enlarge the domain of observation of the dynamic behavior of polymeric systems by applying the time-temperature superposition principle to the rheological spectra. This has been done for gluten subfractions (Tsiami et al 1997a). Our previous studies showed that the rheological response of gluten to heating was complex (Lefebvre et al 1994b). It depended on the temperature range examined and on SH status. The height of the elastic plateau decreased by 10× when temperature was increased from 10 to 55°C, but at >55°C, an increased height of the elastic plateau was associated with enhanced protein aggregation. Such temperature-dependent behavior has been described previously (Attenburow et al 1990). However, the height of the elastic plateau remained almost constant over the whole temperature range (5–80°C) when N-ethylmaleimide (NEMI), an SH-blocking agent, was added to gluten (Lefebvre et al 1994b). In addition, whether SH groups were blocked or not, the characteristic frequency associated with the plateau was shifted toward higher values when temperature was increased from 10 to 80°C, as was the local mobility of polypeptide chains (Lefebvre et al 1994b, Hargreaves et al 1995b).

The objective of the present work was to examine the reversibility or irreversibility of the effects of heat treatments on gluten viscoelasticity. The influence of HMW-GS on gluten response was considered by analyzing four near-isogenic lines with varying HMW-GS composition.

MATERIALS AND METHODS

Wheat Lines

A set of four wheat flour lines with different alleles at the *Glu-1* loci, which codes for HMW-GS, was used throughout this study. They were obtained by crosses between genotypes Olympic and Gabo (Lawrence et al 1998) and kindly provided in 1994 by R. B. Gupta and G. J. Lawrence, CSIRO (division Plant Industry, Can-

berra, Australia). They include one standard (control) line with five subunits (1, 17+18, 5+10) controlled by the *Glu-A1, Glu-B1,* and *Glu-D1* loci, respectively. The three other lines were null at two or three of these loci: *Glu-A1/Glu-B1* null contained subunits 5+10 only, *Glu-A1/Glu-D1* null contained subunits 17+18 only, and triple null (*Glu-A1/Glu-B1/Glu-D1* null) had no HMW-GS. The flours were stored at >4°C before gluten extraction.

Gluten Extraction

Flour was extracted first by 0.05*M* phosphate buffer, 0.1*M* KCl, 0.05*M* EDTA, pH 7.8, with 2% Triton X114 to remove lipids and nonstorage proteins (Hargreaves et al 1995a). After centrifugation (5,000 × *g*, 30 min), the pellet containing starch and storage proteins was extensively washed with deionized water and the gluten was recovered and then freeze-dried. Protein contents (N × 5.7) of control, *1A/1B* null, *1A/1D* null, and triple null lines were 76.5, 71.1, 70, and 83%, respectively.

Size Distribution of Gluten Proteins

Glutens were recovered from the rheometer (see below) before and after heat treatment and were submitted to a sequential two-step extraction procedure and then to size-exclusion (SE) HPLC (Hargreaves et al 1996). The gluten samples were first suspended for 2 hr in 0.5% SDS 12.5 m*M* borate buffer, pH 8.5, and centrifuged (30,000 × *g*, 20 min), yielding extractable and unextractable fractions. The unextractable fraction was suspended overnight in 2% SDS borate buffer and sonicated (6W, 30 sec). After centrifugation (30,000 × *g*, 20 min), the supernatant was recovered. The supernatants from each extraction step were submitted to SE-HPLC as described previously (Cornec et al 1994). The chromatograms of the proteins of both extractions were integrated into three zones corresponding to large-size glutenin polymers (P1 > 500,000), medium-size glutenin polymers (500,000 > P2 > 70,000), and monomeric gliadins (P3 < 70,000). In the experiments reported here, extractions and chromatography were performed singly because of the quantity of material available. But independent analyses showed that the relative standard error of the determination of prolamin fractions was <5%.

Heat Treatment of Gluten Samples

Gluten samples for the experimental study were prepared at 20°C as described previously (Cornec et al 1994). In the first series of experiments, distilled water was used to rehydrate the freeze-dried materials; in the second series, a 0.1*M* NEMI solution was used instead of water. After a rest period of 1 hr at 20°C, the rehydrated samples were transferred into the measuring device of the rheometer maintained at 20°C. The samples were covered with distilled water or with 0.1*M* NEMI solution to prevent them from drying out during the experiments and to ensure full hydration at any temperature. Before starting the experiments, the samples were rested 1 hr at 20°C to allow for stress dissipation.

Two experimental sequences were conducted. In the first experimental sequence, samples were submitted to two successive temperature cycles in situ in the measuring device of the rheometer. One cycle had a linear temperature ramp from 20 to 40°C in 1 hr, a plateau at 40°C maintained for 0.5 hr, and a descending ramp back to 20°C. The second cycle had a linear ramp from 20 to 70°C in 2.5 hr, a plateau at 70°C maintained for 15 hr, and a descending ramp to 20°C. In the second experimental sequence, the first temperature plateau was maintained long enough (≈5 hr) to allow recording the mechanical spectrum at 40°C.

Dynamic Rheological Measurements

The rheometer (CSL 100, Carri-Med Ltd., Dorking, Surrey, England) used a cone and plate geometry (cone angle: 4°, cone diameter: 2 cm). Measurements were performed in the dynamic regime, with a strain amplitude sufficiently low (≈3%) to assume that the viscoelastic behavior remained within the linear region under test conditions (Cornec et al 1994, Lefebvre et al 1994b). Two types of tests were conducted: 1) measurements at fixed frequency (f = 1 Hz) to monitor changes in the samples with time; 2) frequency sweeps at a fixed temperature, during which the frequency was decreased from 36 to 0.001 Hz (≈5 hr) to characterize the viscoelastic response, which is recorded as the variations of the storage modulus G' and the loss modulus G'' versus the frequency f (mechanical spectrum).

Test 2 was recorded first at 20°C before starting the first temperature cycle (initial spectra), then at 20°C at the end of this cycle, and then again at the end of the second cycle (final spectra). Test 1 was recorded during the temperature plateau phases at 40 and 70°C. For the 70°C plateau, monitoring of G' and G'' versus time continued for 10 hr and was followed by recording the mechanical spectrum at 70°C (≈5 hr). In addition, G' and G'' were measured at 1 Hz for 20 min before recording the final 20°C spectra (to check the stability of the samples at 20°C after the heat treatment) and during the temperature ramps.

Analysis of Mechanical Spectra

The basis of the analysis of the mechanical spectra of gluten was reported previously (Cornec et al 1994, Lefebvre et al 1994b). The results were converted in terms of the components of the complex compliance (storage compliance J' and loss compliance J''), although it contains exactly the same rheological information as the complex modulus (G' and G''), because it is more easily amenable to quantitative treatment. The mechanical spectra of gluten display a loss compliance peak, indicative of a network-type structure, the maximum of which is usually located within the experimental frequency window at 5–70°C. In the region of the peak, the rheological functions $J'(f)$ and $J''(f)$ can be fitted by Cole-Cole functions:

$$J'(f) - J_g = \left(J_N^0 - J_g\right)\frac{\left[\left(f_o/f\right)^n + \cos(\pi n/2)\right]}{\left[\left(f_o/f\right)^n + 2\cos(\pi n/2) + \left(f/f_o\right)^n\right]} \quad (1)$$

$$J''(f) = \left(J_N^0 - J_g\right)\frac{\sin(\pi n/2)}{\left[\left(f_o/f\right)^n + 2\cos(\pi n/2) + \left(f/f_o\right)^n\right]} \quad (2)$$

where J_N^0 is the compliance associated with the viscoelastic plateau, the high frequency limit of which is marked by the loss peak; f_0 is the frequency of the maximum in J'' (central frequency of the retardation process considered); the exponent n is the frequency spread parameter that measures the broadness of the retardation time distribution corresponding to the loss peak; and J_g is the glassy compliance that can be ignored in favor of the J' and J_N^0 values. The modulus corresponding to the viscoelastic plateau is: $G_N^0 = 1/J_N^0$ and G_N^0 can be taken as a measure of network elasticity.

In previous reports (Cornec et al 1994, Lefebvre et al 1994b), the parameters J_N^0, f_0 , and n were obtained by fitting data directly to Equations 1 and 2. However, it seems indeed preferable to follow the classical procedure, which plots J' against J''. On such plots (Cole-Cole plots), the retardation process shows as an arc of circle passing through the origin, the equation of which is obtained by elimination of f/f_0 between Equations 1 and 2:

$$J'' = \left(\frac{J_N^0}{2\tan(\pi n/2)}\right)\left[\left(1 + 4\frac{J'}{J_N^0}\left(1 - \frac{J'}{J_N^0}\right)\tan^2(\pi n/2)\right)^{\frac{1}{2}} - 1\right] \quad (3)$$

The fit of Equation 3 to the results gives the Cole-Cole parameters J_N^0 and n, and a simple geometrical construction yields f_0 (Tschoegl 1989).

RESULTS

Viscoelastic Behavior of Gluten from Near-Isogenic Lines at 20°C Before Heating

Figure 1A shows the initial mechanical spectra at 20°C of glutens from near-isogenic lines rehydrated with a 0.1*M* NEMI solution, plotted as variations of the storage and loss moduli (G' and G'', respectively) against the frequency. The spectra obtained without NEMI were practically identical (Fig. 1B), from which we can conclude that the SH-blocking agent does not modify the viscoelastic behavior of the glutens in such conditions.

The spectra of the control and the 5+10 lines were very close each other, whereas the spectrum of the 17+18 line, similar in shape, displayed much lower values of both moduli over the frequency range. All three were of the usual type observed for glutens. As discussed previously (Cornec et al 1994, Lefebvre et al 1994), the frequency window spans the viscoelastic plateau, which extends beyond both frequency limits.

The spectrum of the triple null sample was the same type but besides showing very low values of G' and G'', the viscoelastic plateau was shifted in this case to lower frequencies so that only its high-frequency extremity, marked by the intersection of the moduli G' and G'', remained visible within the frequency window, which now encompassed the beginning of the transition zone from the rubbery to the glassy behavior. We obtained very similar results on fractions low in large glutenin polymers extracted by dilute HCl from different glutens using a sequential extraction process (Cornec et al 1994, Popineau et al 1994a).

The Cole-Cole plots corresponding to some of the spectra of Fig. 1 are shown on Fig. 2 as examples. On such plots, the closer the points to the origin, the higher the corresponding frequencies. Figure 2 illustrates how difficult it is to extract meaningful rheological quantities from the mechanical spectra of glutens. In effect, with the exception of the triple null line, the arc of the circle corresponding to the high frequency loss peak was defined by a small number of experimental points, all situated on the ascending part of the arc, and strongly overlapped at higher J' and J'' values by slower retardation mechanisms. For the initial 20°C spectra of the triple null line (with and without NEMI), the arc of the circle was much better defined (Fig. 2), but because the characteristic frequency was very close to the lower limit of the experimental range, the uncertainty of its value was large.

TABLE I
Reproducibility of Rheological Characterization of Gluten at 20°C by Dynamic Measurements[a,b]

Number of Samples Tested	Cole-Cole Parameters[c]		
	G_N^0 (N m^{-2})	f_0 (Hz)	*n*
10	880 (60)	0.05 (0.04)	0.41 (0.01)

[a] Samples (17+18 line) hydrated with 0.1*M* N-ethylmaleimide (NEMI), an SH-blocking agent; ascending frequency sweep 0.001–36 Hz.
[b] Average values with standard deviations in parentheses.
[c] G_N^0 = modulus corresponding to the viscoelastic plateau; f_0 = characteristic frequency; *n* = frequency spread.

In an independent study, we analyzed the spectra recorded directly at 20°C of 10 samples prepared with NEMI from 17+18 lines. The procedure followed was the same, except that the frequency sweep was operated in the ascending frequency mode. The results in Table I show that fair reproducibility was achieved for the height of the viscoelastic plateau G_N^0 and for the spread parameter *n*, but that the uncertainty in the determination of the characteristic frequency f_0 was very large: the relative standard errors were 2.3, 1.0, and 26%, respectively. Similar values were found for the relative standard errors of the glutens of the control (four samples), 5+10 (three samples), and triple null lines (four samples) analyzed in the same conditions. In such rheological analyses, the scatter of the results stems a priori from three causes: defect in the reproducibility of the preparation and the mechanical history of the samples; measurement errors in the determination of G' and G'' (or J' and J''); and, finally, errors involved in processing of the instrumental data (here, in the determination of the Cole-Cole parameters). The second cause is surely a minor one when working with this type of material. The third cause is probably the main one, at least as to the uncertainty in the determination of f_0, for the reasons given above. Because of the limited amount of material available, we did not repeat the reproducibility study on the heat-treated samples. But it seems reasonable to assume that for all spectra, the relative standard errors that affect the rheological parameters were of the same magnitude as those reported above; thus, the 5% confidence intervals on G_N^0 , *n*, and f_0 are 5, 2, and 60%, respectively.

Table II shows that, within the frequency range considered, four points must be considered:

Point 1. NEMI had little effect, if any, on the viscoelastic behavior of gluten; when the values of the rheological parameters with and without NEMI differed, the difference was hardly significant on statistical grounds and has no particular importance from a rheological point of view.

Point 2. The control and 5+10 lines showed viscoelastic properties that were very close to each other; the differences in the values of the Cole-Cole parameters were within the range of experimental uncertainty.

TABLE II
Cole-Cole Parameters Analyzing Initial Mechanical Spectra of Glutens from Different Near-Isogenic Lines at 20°C[a,b]

Line	Cole-Cole Parameters[c]		
	G_N^0 (N m^{-2})	f_0 (Hz)	*n*
Control	3,330 (3,210)	0.20 (0.6)0	0.324 (0.349)
5+10	3,660 (3,130)	0.65 (0.36)	0.400 (0.398)
17+18	1,520 (1,140)	0.27 (0.28)	0.420 (0.446)
Triple null	14.3 (18.8)	<0.001 (<0.001)	0.639 (0.600)

[a] Descending frequency sweeps 36–0.001 Hz.
[b] Values for samples hydrated with 0.1*M* N-ethylmaleimide (NEMI), an SH-blocking agent, with values for samples hydrated with water in parentheses.
[c] G_N^0 = modulus corresponding to the viscoelastic plateau; f_0 = characteristic frequency; *n* = frequency spread. Confidence (5%) intervals for Cole-Cole parameters: ±0.05 G_N^0, ±0.6 f_0 , and ±0.02 *n*, respectively.

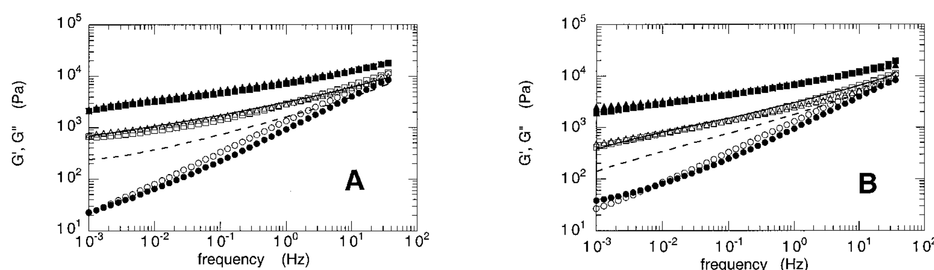

Fig. 1. Mechanical spectra of gluten samples from control (▲, Δ), 5+10 (■, □), 17+18 (——, – – –), and triple null lines (●, ○) recorded at 20°C before temperature cycles (initial spectra). Samples of freeze-dried glutens rehydrated with 0.1*M* N-ethylmaleimide (NEMI) solution (**A**) or with distilled water (**B**). Measurements taken under 0.03 strain amplitude. Black symbols or continuous line: storage modulus G'. White symbols or dotted line: loss modulus G''.

Point 3. The main difference between the control and 5+10 lines as compared with the 17+18 lines was that the height of the viscoelastic plateau was two times lower for 17+18 than for control and 5+10.

Point 4. The triple null line differed from the control and from 5+10 and 17+18 in all three parameters. The height of the viscoelastic plateau was dramatically decreased, and the position of this plateau was shifted to lower frequencies by more than two logarithmic decades; also, the width of the associated loss peak decreased significantly.

Point 1 indicates that SH-SS interchange reactions were not responsible for the viscoelastic response of gluten, but this does not mean that the status of the SH and SS groups had no effect on the viscoelastic properties. The global conclusion we can draw from points 2–4 is that HMW-GS are practically indispensable to the manifestation of gluten viscoelasticity, their absence results in a breakdown of gluten rheology (point 4), but they are not equivalent to each other with respect to viscoelastic potential. The double deletion of *1A/1B* loci did not seem to have appreciable consequences on gluten viscoelasticity in the plateau region (point 2). On the contrary, the double deletion *1A/1D* resulted in a considerable drop in the height of the viscoelastic plateau (point 3). However, the amplitude of the effect of this double deletion seemed to depend on the genetic background because it was less pronounced in the case of the *1A/1D* null (–, 7+9, –) Sicco line (Popineau et al 1994a).

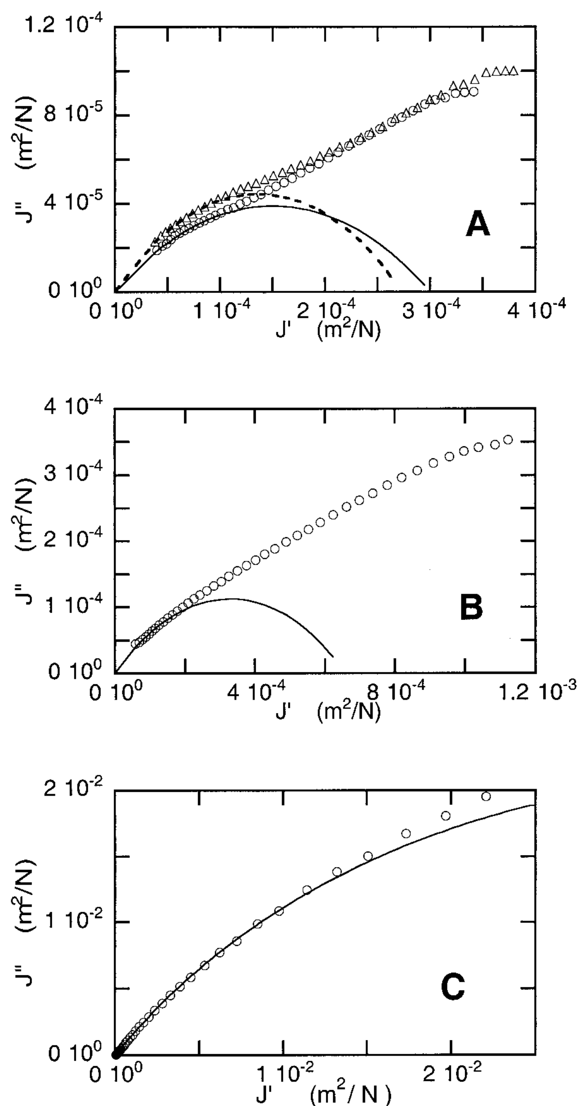

Fig. 2. Data from Fig. 1A (glutens rehydrated with 0.1*M* NEMI) in the complex compliance plane ($J'(f) - J''(f)$). **A,** Control (○ and ——) and 5+10 (Δ and – – –) lines. **B,** 17+18 line. **C,** triple null line. Solid and dotted lines show the Cole-Cole arcs of circle obtained by fitting Eq. 3 to experimental points in the high frequency region of the spectra.

One fact is intriguing in the viscoelastic behavior of gluten: the decrease in f_0 parallels the decrease in the height of the viscoelastic plateau, whereas f_0 and G_N^0 vary in an opposite way in classical transient networks such as entangled polymer solutions. We observed the same peculiarity for gluten fractions obtained by a sequential extraction procedure (Cornec et al 1994). It is probably a feature related to the multilevel structure of gluten systems.

Another aspect of the complexity of the rheological behavior of gluten is illustrated by the comparison of the results reported for the 17+18 line (hydrated with 0.1*M* NEMI) in Tables I and II. At least for G_N^0, these results differed significantly. The value of G_N^0 obtained from the spectra recorded in the ascending frequency sweep mode (Table I) was higher than the value obtained in the descending frequency sweep mode (Table II). In the first case, the sample was submitted to low frequency strains (i.e., solicitations involving larger flow contributions as compared with high frequency strains) for a longer time than in the second case due to the way stress-controlled rheometers operate in the dynamic regime in the controlled strain amplitude mode. Gluten shows some degree of strain hardening induced by flow, and this degree depends on the line or the cultivar (J. Lefebvre, *unpublished results*). This issue will be discussed in a subsequent report on linearity.

20–40–20°C Temperature Cycles

When the temperature of the samples was increased from 20 to 40°C, the moduli G' and G'' measured at 1 Hz monotonously decreased; no hysteresis was observed upon cooling back to 20°C for samples hydrated with 0.1*M* NEMI and it was quite small for samples that did not contain NEMI. This is illustrated in Fig. 3 for control and triple null lines. The variations of G' and G'' followed similar curves for all lines, but their amplitude depended on the line and on the presence of NEMI. With NEMI, heating from 20 to 40°C resulted in a 30% decrease of G' for the control and 5+10 lines and a 70% decrease of G' for the triple null line. Without NEMI, G' dropped by 35, 45, 60, and 68% for the control, 5+10, 17+18, and triple null lines, respectively. However, the amount of information that temperature cycling experiments contain is quite small, and they can actually be misleading because temperature changes shift the mechanical spectra along both the moduli and the frequency axes, so that measurements at one frequency do not allow sound conclusions to be drawn about the way temperature affects the viscoelastic behavior.

Either with or without NEMI, the spectra recorded at 20°C after cycling to 40°C were very similar to those recorded before cycling (Fig. 4 shows control and triple null lines). The analysis of the spectra obtained with NEMI confirmed that heating to 40°C induced few

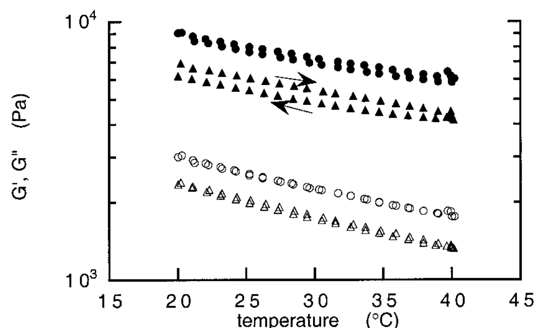

Fig. 3. Variation of the storage (G') and loss (G'') moduli measured at the frequency f = 1 Hz under a 0.03 strain amplitude during a 20–40–20°C temperature cycle. Results obtained from two control line gluten samples rehydrated with distilled water (▲, Δ) and with 0.1*M* N-ethylmaleimide (NEMI) (●, ○). Black symbols: G'. White symbols: G''. Arrows indicate direction of temperature ramp.

irreversible changes in the viscoelastic behavior of gluten (Table III). Moreover, at least a part of the significant but limited increase in G_N^0 at 20°C, which was the main effect of the temperature cycle, resulted from the shear-induced irreversible changes we mentioned earlier. Without NEMI, the changes in the Cole-Cole parameters induced by the temperature cycle remained relatively small or insignificant for the control, 5+10, and triple null lines (Table III). For the 17+18 line without NEMI, an appreciable decrease was observed in G_N^0 and f_0 that remains unexplained.

With the exception of the 17+18 line without NEMI, we can conclude that heating gluten to 40°C entails no irreversible changes in its structure resulting in important rheological consequences as far as it can be inferred from the direct comparison of the mechanical spectra in the 10^{-3} to 40 Hz frequency range. The variations in dynamic moduli observed as temperature increased from 20 to 40°C were basically a normal temperature effect on the viscoelasticity of the gluten network. This effect, reversible and independent of the time during which the temperature was maintained, did not involve any chemical change, with or without NEMI.

A detailed study and discussion of the normal (reversible) effect of temperature on gluten viscoelasticity is not within the scope of this article. Nevertheless, we want to point out a general trend. Figure 5A shows that at 40°C, the dissipation processes marking the high frequency limit of the viscoelastic plateau were more clearly individualized than at 20°C for the control line with NEMI. Comparison of the results in Table IV with those in Table III shows the reversible effect of temperature. The characteristic frequency f_0 was shifted to a significantly lower value, whereas the G_N^0 was substantially reduced (Table IV). The same applies to the 5+10 and 17+18 lines and is also relevant to the control, 5+10, and 17+18 lines rehydrated in distilled water (Table IV). The gluten from the 17+18 line was especially sensitive to the effect of temperature. In glutens from the triple null line (Fig. 5B), f_0 was shifted to a higher value at 40°C as compared with 20°C and G_N^0 was not substantially decreased (Table IV), probably because the irreversible changes responsible for the noticeable increase in the 20°C value after heating to 40°C (Tables II and III) compensated in this case for the normal temperature-induced drop in the height of the viscoelastic plateau.

20–70–20°C Temperature Cycles

The manner in which the glutens behaved when submitted to temperatures >50°C differed completely depending on whether

TABLE III
Cole-Cole Parameters Analyzing Mechanical Spectra of Glutens from Different Near-Isogenic Lines at 20°C After the First Temperature Cycle[a,b]

	Cole-Cole Parameters[c]		
Line	G_N^0 (N m^{-2})	f_0 (Hz)	n
Control	4,680 (3,250)	0.80 (0.66)	0.366 (0.390)
5+10	4,380 (2,720)	0.72 (0.65)	0.408 (0.457)
17+18	1,630 (480)	0.45 (0.04)	0.446 (0.461)
Triple null	28.6 (66.5)	<0.001 (0.004)	0.584 (0.603)

[a] First temperature cycle: 20→40→20°C. Descending frequency sweeps 36–0.001 Hz.
[b] Values for samples hydrated with 0.1M N-ethylmaleimide (NEMI), an SH-blocking agent, with values for samples hydrated with water in parentheses.
[c] G_N^0 = modulus corresponding to the viscoelastic plateau; f_0 = characteristic frequency; n = frequency spread. Confidence (5%) intervals for Cole-Cole parameters: ±0.05 G_N^0, ±0.6 f_0, and ±0.02 n, respectively.

TABLE IV
Cole-Cole Parameters Analyzing Mechanical Spectra of Glutens from Different Near-Isogenic Lines at 40°C[a,b]

	Cole-Cole Parameters[c]		
Line	G_N^0 (N m^{-2})	f_0 (Hz)	n
Control	2,000 (1,440)	0.035 (0.11)	0.257 (0.300)
5+10	nd (1,260)	nd (0.24)	nd (0.376)
17+18	620 (120)	0.064 (0.006)	0.365 (0.443)
Triple null	50 (56)	0.014 (0.011)	0.617 (0.613)

[a] Descending frequency sweeps 36–0.001 Hz.
[b] Values for samples hydrated with 0.1M N-ethylmaleimide (NEMI), an SH-blocking agent, with values for samples hydrated with water in parentheses; nd = not determined.
[c] G_N^0 = modulus corresponding to the viscoelastic plateau; f_0 = characteristic frequency; n = frequency spread. Confidence (5%) intervals for Cole-Cole parameters: ±0.05 G_N^0, ±0.6 f_0, and ±0.02 n, respectively.

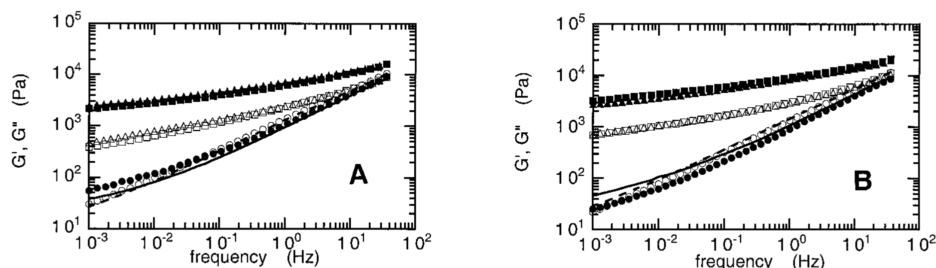

Fig. 4. Mechanical spectra at 20°C before and after heating to 40°C (1st temperature cycle) for control and triple null lines rehydrated with distilled water (**A**) and with 0.1M N-ethylmaleimide (NEMI) (**B**). Strain amplitude: 0.03. Control line before heating (▲, Δ) and after heating to 40°C (■, □). Triple null line before heating (●, ○) and after heating to 40°C (——). Black symbols or continuous line: storage modulus G'. White symbols or dotted line: loss modulus G''.

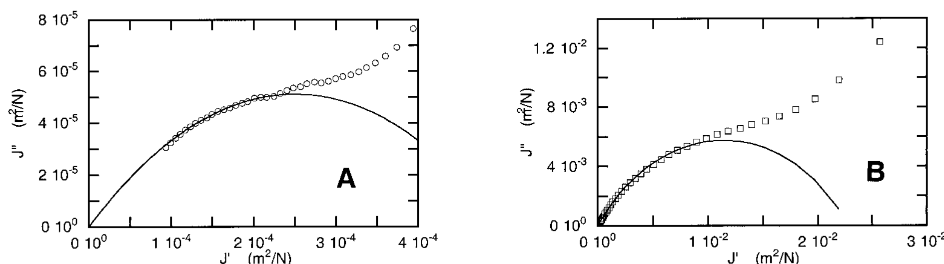

Fig. 5. Mechanical spectra at 40°C plotted in the complex plane $(J'(f) - J''(f))$. **A,** control line rehydrated with 0.1M N-ethylmaleimide (NEMI); **B,** triple null line rehydrated with 0.1M NEMI. Strain amplitude: 0.03. Solid lines show the Cole-Cole arcs of circle obtained by fitting Eq. 3 to experimental points in the high frequency region of the spectra.

they had been prepared with or without NEMI. This is illustrated by the variations of G' and G'' with time during the temperature plateau at 70°C. Figure 6 shows that without NEMI, heating the standard Gabo line gluten at 70°C for 10 hr increased G' (measured at 1 Hz) by 5×, whereas G'' measured at this frequency did not vary appreciably. The variation of G' slackened as time elapsed but continued after 10 hr. Therefore, the mechanical spectrum recorded at 70°C after the 10-hr period has no meaning. Glutens of the other lines display the same type of kinetics at 70°C, with still larger variations of G' for 17+18 and triple null lines. There was an appreciable dependence of G'' on time in the triple null lines (Fig. 7).

On the contrary, when prepared with NEMI, G' and G'' decreased gradually to a limited extent during heating at 70°C for the control, 5+10, and 17+18 lines (Fig. 6). However, for the triple null line, G' increased slightly (Fig. 7). In fact, after 10 hr at 70°C, G' had almost reached a plateau in all cases, as indicated by a few complementary experiments during which the samples were kept at 70°C for a longer time. Consequently, the frequency sweeps at 70°C following the heating period can be considered to yield true mechanical spectra characterizing the viscoelastic response at 70° of the glutens prepared with NEMI.

After cooling the heated samples to 20°C, the moduli stabilized immediately to values strictly constant with time. By comparing the mechanical spectra recorded after cooling to those recorded at 20°C before heating to 70°C, we can measure the amplitude of the irreversible changes induced by the second temperature cycle. The results of the analysis of the final 20°C spectra are given in Table V and some of these spectra are shown in Fig. 8. Comparison of the data in Table V with those in Table III demonstrates that with NEMI, noticeable irreversible modifications affecting essentially G_N^0 occurred in the glutens during the heating period, except in the control line which was left unchanged. The distribution width of the retardation times (spread parameter n) was almost unaffected, whereas the variations of the characteristic frequency f_0 were probably not significant, except for the triple null line where a tenfold increase of this parameter was observed. Part of the increase in G_N^0 may be due to shear hardening as discussed above, but such an effect alone cannot explain the rather large changes measured for the 5+10, 17+18, and especially the triple null lines (quite small modifications resulted from the first temperature cycle), and they should also have been observed for the control line if it had resulted from strain hardening. It is likely, therefore, that some irreversible temperature-induced changes occurred in glutens heated at 70°C with NEMI. Their amplitude depended on the HMW-GS. Their nature remains unknown but, because of the SH-blocking agent, they should not involve SH-SS reactions.

Without NEMI, the irreversible changes induced by heating at 70°C affected all three Cole-Cole parameters. When compared with the 20°C spectra values before the second temperature cycle, the final 20°C spectra values of G_N^0 and f_0 were increased tremendously, whereas n showed a marked decrease, indicating a sharpening of the loss peak after the 70°C heat treatment (Tables V and III). As a result, after heating at 70°C without NEMI, gluten becomes a completely different material as far as its rheological properties are considered. Also, without NEMI, the amplitude of the effect depended on the line. The lines ranked in the increasing order were: control < 5+10 < 17+18 < triple null, with G_N^0 multiplied by a factor of 10, 15, 38, and 240, respectively. The effect was especially remarkable for the triple null line because the 70°C heat treatment raised its viscoelasticity to a level comparable to that of the heat-treated glutens of the other lines (Fig. 8A and C).

Comparing the effects of the heat treatment with and without NEMI on gluten viscoelasticity at 20°C demonstrated that, by far, the largest part of the rheological changes observed without NEMI

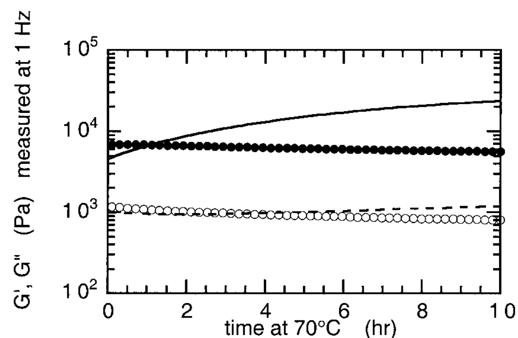

Fig. 6. Variations with time of the storage (G') and loss (G'') moduli measured at 1 Hz under 0.03 strain amplitude while heating at 70°C. Control line rehydrated with 0.1*M* N-ethylmaleimide (NEMI) (●, ○) or with distilled water (——, – – –). Black symbols or continuous line: G'. White symbols or dotted line: G''.

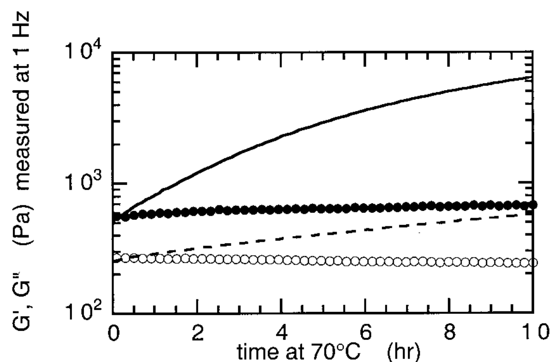

Fig. 7. Variations with time of the storage (G') and loss (G'') moduli measured at 1 Hz under 0.03 strain amplitude while heating at 70°C. Triple null line rehydrated with 0.1*M* N-ethylmaleimide (NEMI) (●, ○) or with distilled water (——, – – –). Black symbols or continuous line: G'. White symbols or dotted line: G''.

TABLE V
Cole-Cole Parameters Analyzing Mechanical Spectra of Glutens from Different Near-Isogenic Lines at 20°C After the Second Temperature Cycle[a,b]

	Cole-Cole Parameters[c]		
Line	**G_N^0 (N m^{-2})**	**f_0 (Hz)**	**n**
Control	5,000 (31,900)	0.80 (28)	0.372 (0.312)
5+10	7,700 (41,000)	1.2 (10)	0.386 (0.315)
17+18	2,700 (18,400)	0.24 (5.0)	0.424 (0.349)
Triple null	760 (16,000)	0.11 (5.0)	0.504 (0.384)

[a] Second temperature cycle: 20→70→20°C. Samples submitted to temperature of 70°C for an overall period of ≈15 hr. Descending frequency sweeps 36–0.001 Hz.
[b] Values for samples hydrated with 0.1*M* N-ethylmaleimide (NEMI), an SH-blocking agent, with values for samples hydrated with water in parentheses.
[c] G_N^0 = modulus corresponding to the viscoelastic plateau; f_0 = characteristic frequency; n = frequency spread. Confidence (5%) intervals for Cole-Cole parameters: ±0.05 G_N^0, ±0.6 f_0, and ±0.02 n, respectively.

TABLE VI
Cole-Cole Parameters Analyzing Mechanical Spectra of Glutens from Different Near-Isogenic Lines at 70°C Rehydrated with 0.1*M* NEMI[a]

	Cole-Cole Parameters[b]		
Line	**G_N^0 (N m^{-2})**	**f_0 (Hz)**	**n**
Control	4,300	18	0.348
5+10	6,700	40	0.377
17+18	1,850	32	0.453
Triple null	450	1.8	0.522

[a] Spectra recorded after samples were maintained at 70°C for 10 hr following the first temperature cycle. Descending frequency sweeps 36–0.001 Hz.
[b] G_N^0 = modulus corresponding to the viscoelastic plateau; f_0 = characteristic frequency; n = frequency spread. Confidence (5%) intervals for Cole-Cole parameters: ±0.05 G_N^0, ±0.6 f_0, and ±0.02 n, respectively.

resulted from chemical reactions affecting the SS bonds of HMW-GS by a few SH groups present in gluten. Comparison of the spectra recorded at 70°C with NEMI with the final 20°C spectra gives the measure of the rheological temperature effect because: 1) the irreversible temperature-induced changes in the structure of the glutens with NEMI were practically completed before the spectra were recorded at 70°C, and 2) cooling the heat-treated glutens to 20°C did not cause any more change.

The 70°C spectra of the glutens with NEMI were are all qualitatively similar and did not differ from those of the final 20°C spectra but they were somewhat low on the moduli scale (Fig. 8). The results of the Cole-Cole analysis of these spectra are given in Table VI. Compared with those reported in Table V for the final 20°C spectra with NEMI, we see that G_N^0 at 70°C was lower. Values were 86, 88, 70, and 59% of values at final 20°C spectra for the control, 5+10, 17+18, and triple null lines, respectively. On the contrary, the f_0 values were higher at 70°C: ≈20, 30, 30, and 15 times larger for the control, 5+10, 17+18, and triple null lines, respectively, than those obtained from the final 20°C spectra. The spread parameter n was not much affected by temperature although the differences were significant. The fact that G_N^0 was substantially lower at 70°C than at 20°C confirmed that the height of the viscoelastic plateau of the glutens was a decreasing function of temperature as already stressed when comparing the results at 40°C with those obtained at 20°C. This means that gluten viscoelasticity is not of the classical rubbery type governed by entropy, but includes an enthalpic contribution that probably involves hydrogen bonds participating in the connectivity of the network. A corollary is that one is not allowed to apply the time-temperature superposition principle to gluten, even if master curves can be seemingly obtained by shifting the mechanical spectra recorded at different temperatures along both axes. As to the temperature dependence of f_0, the direction of the shift was seemingly opposite to that observed on cooling from 40 to 20°C for the control, 5+10, and triple null lines, which would mean that at 20–70°C, temperature affects several structural mechanisms that do not respond in the same way. However, this requires confirmation by a detailed study because the f_0 shifts observed during the first temperature cycles are quite small considering the errors involved in the determination of the characteristic frequency.

Effect of Heating on Size Distribution of Gluten Proteins

The gluten samples submitted to the 20–70–20°C temperature cycles were recovered after the rheological study and analyzed by SE-HPLC after sequential extraction. Extractability in 0.5 and 2% SDS buffers and size distributions were compared with those of unheated glutens (Table VII). Although single determinations were made, the differences observed were significant considering the reproducibility of the procedure, which had been checked as explained above.

As expected, the extractability of unheated gluten proteins and their size distribution depended on the HMW-GS composition of the lines. The control line comprised the lowest amount of proteins extracted in 0.5% SDS borate buffer, and the triple null line comprised the largest amount (gluten protein from this line was almost completely extractable). Furthermore, gluten proteins from the 17+18 line were more extractable than those from the 5+10 line. Accordingly, the control line showed the highest amount of larger size glutenin polymers (P1 fraction extracted with 2% SDS and sonication), and the triple null line the lowest. Again, glutenin polymers from the 5+10 line were more extensively aggregated than those from the 17+18 line. In addition to these differences in glutenin composition, the 17+18 and triple null lines contained larger amounts of monomeric gliadins (P3). All these characteristics agree with the rheological behavior of the four glutens, fewer aggregated glutenin polymers is related to lower viscoelasticity (Cornec et al 1994, Popineau et al 1994a, Gupta et al 1995, Hargreaves et al 1996).

Analyses performed at 20°C on glutens after thermal treatment at 70°C with NEMI revealed changes in extractability and size distribution for the 17+18 and triple null lines. Proteins extracted in 0.5% and 2% SDS buffers decreased at the benefit of an unextractable residue fraction. In control and 5+10 lines, no residue was formed, but less glutenin polymer was extracted in 0.5% SDS buffer and more was recovered in 2% SDS buffer, indicating that even when SH groups were blocked, heating at 70°C had enhanced glutenin aggregation. Heating without NEMI modified more extensively

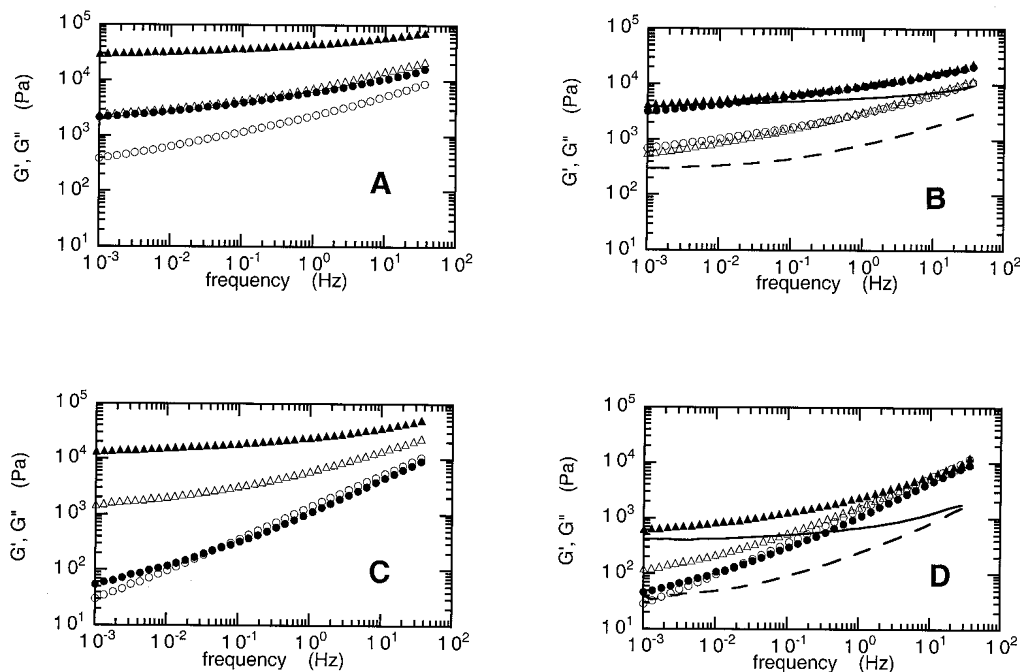

Fig. 8. Mechanical spectra of gluten samples recorded before and after heating at 70°C (2nd temperature cycle) for the control and triple null lines rehydrated with distilled water (**A** and **C**) and with 0.1*M* N-ethylmaleimide (NEMI) (**B** and **D**). Strain amplitude: 0.03. At 20°C before the start of the 2nd temperature cycle (●, ○). At 20°C after heating at 70°C and cooling to 20°C (▲, Δ). At 20°C , for samples rehydrated with NEMI maintained at 70°C for 10 hr (——, – – –). Black symbols or continuous line: storage modulus G'. White symbols or dotted line: loss modulus G''.

the properties of gluten proteins of all four lines. First, the total amount of proteins that were extracted by the sequential procedure was reduced. Protein residues account for 68, 34, 47, and 39% of the total proteins from the control, 5+10, 17+18, and triple null lines, respectively, whereas no residue was observed with unheated glutens. Contents of monomeric proteins and glutenin polymers extracted in 0.5% SDS buffer also decreased. The fate of glutenin polymers extracted with 2% SDS buffer depended on the lines: they decreased in the control line, were stable in the 5+10 line, but increased in the 17+18 and triple null lines. These results are indicative of different aggregative potential of the glutenin polymers depending on their HMW-GS composition. Those containing the 5+10 subunits aggregated as an unextractable residue only under heating without NEMI. Independent experiments had shown that residue proteins resulting from heating could be extracted only after addition of reducing agent. This implies that SS bridges were involved in the heat-induced unextractability through SH-SS interchanges resulting in covalent cross-linking of glutenin aggregates. When only subunits 17+18 or no HMW subunits were present, some aggregation occurred even with NEMI. The cause of this particular behavior is not known.

The relationship between gluten polymer composition and gluten rheology is illustrated in Fig. 9, where the height of the viscoelastic plateau is plotted on log-log scales against the cumulated fractions of P1 and of the residue for the unheated and heated glutens. The results follow reasonably well ($R = 0.7$) a power law (exponent 2.6) with the exception of the point relative to the unheated triple null gluten. After heating at 70°C, this sample was nevertheless normalized.

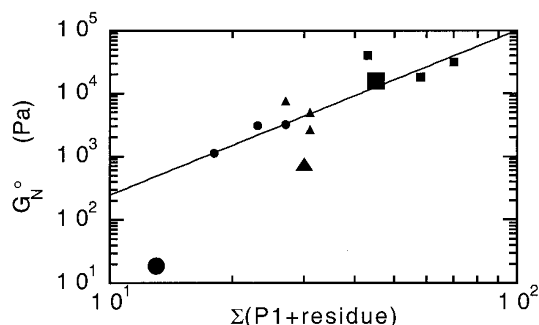

Fig. 9. Correlation between the height of the viscoelastic plateau at 20°C and the proportion Σ(P1+residue) (%) of the largest glutenin aggregates determined by the fractionation procedure on unheated samples (●), samples rehydrated with 0.1*M* N-ethylmaleimide (NEMI) heated at 70°C (▲), and samples rehydrated with distilled water heated at 70°C (■). Larger symbols correspond to the triple null line gluten samples. Line is the power law fit (exponent 2.6, $r = 0.70$) on the experimental points (except the unheated triple null line). Heat treatment, especially with water, normalizes the viscoelastic plateau of the triple null line.

DISCUSSION

Analyses of the viscoelastic behavior of the glutens extracted from near-isogenic lines showed contrasting results. Mechanical properties of glutens depended mainly on HMW-GS composition. Two particular results were noticeable. First, the gluten comprising only subunits 1Dx 5 and 1Dy 10 exhibited the same viscoelasticity as the gluten from the control line with five subunits. This suggests a very high viscoelastic potential for this pair. Second, the gluten comprising the subunits 1Bx 17 and 1By 18 exhibited a lower viscoelasticity than those with subunits 1Dx 5 and 1Dy 10.

This validates the quality scores of HMW glutenin alleles established by Branlard et al (1992) where the pairs 5+10 and 17+18 were rated 30 and 18, respectively, on a scale of 0–30. According to protein composition of the lines, this must arise from structural differences between the subunits, such as additional cysteine residue and regularity of conformation of repetitive domains (Anderson et al 1989, Goldsborough et al 1989, Flavell et al 1989, Shewry et al 1992).

Data from sequential extraction and SE-HPLC explained differences of gluten viscoelasticity. Extractability in 0.5% SDS buffer was larger as viscoelasticity was lower, the amount of largest glutenin polymers (P1 extracted with 2% SDS buffer plus sonication) was greater for higher viscoelasticity. Such a relationship between glutenin polymers and viscoelasticity was observed previously on gluten subfractions as well as on whole glutens from other near-isogenic lines of wheats (Cornec et al 1994, Popineau et al 1994a).

The effect of heating on the four lines was modulated by the range of temperature and by the blocking of SH groups. Whatever the HMW composition of the lines, heating to 40°C resulted in reversible variations of rheological properties, as evidenced by comparison of the rheological results obtained at 20°C before and after the heating-cooling cycle. SH status (free or blocked) did not noticeably influence the behavior in this temperature range. This suggested that possible structural changes in gluten were reversible, that is, they did not involve covalent bonds. In fact, G_N^0 was lower at 40°C than at 20°C. This was in agreement with the disruption of intermolecular H bonds on heating (Belton et al 1994) and with increased mobility of side chains when temperature increases (Har-

TABLE VII
Extractability and Size Distribution of Prolamin in Glutens Submitted to Thermal Treatments With or Without an SH-Blocking Agent (NEMI) (% total protein)[a]

	0.5% SDS				2.0% SDS + Sonication				Residue	Glutenin Extracted	
Line	P1	P2	P3	Σ	P1	P2	P3	Σ	R	0.5% SDS	2.0% SDS
Control											
20°C	5	11	46	62	22	11	5	38	0	16	33
70°C +	4	10	44	58	27	10	5	42	0	14	37
70°C –	1	2	21	24	1	3	4	8	68	3	4
5+10											
20°C	7	13	54	74	16	7	3	26	0	20	23
70°C +	4	11	50	65	23	8	4	35	0	15	31
70°C –	1	4	35	40	18	5	3	26	34	5	23
17+18											
20°C	11	14	59	84	7	4	5	16	0	25	11
70°C +	3	9	49	61	2	7	3	12	26	12	9
70°C –	1	4	31	36	10	4	3	17	47	5	14
Triple null											
20°C	10	20	65	95	3	1	1	5	0	30	4
70°C +	5	12	54	71	7	2	2	11	18	17	9
70°C –	1	6	36	43	15	5	3	23	39	7	20

[a] Glutens were extracted sequentially in 0.5% SDS buffer then 2.0% SDS buffer with sonication. Extracted proteins were subjected to size-exclusion HPLC on Superose 6 (P1 MW > 500,000; P2 500,000 > MW > 70,000; P3 MW < 70,000). + = with NEMI; – = without NEMI.

greaves et al 1995b), which would be expected to weaken the viscoelasticity of the network, and confirmed that intermolecular hydrogen bonds are primarily involved in the association of polypeptide chains in the gluten network (Pézolet et al 1992, Belton 1994).

Heating at 70°C had a more complex effect on glutens. Rheological analyses showed that limited irreversible changes occurred when SH groups were blocked, resulting in increased G_N^0 values after cooling at 20°C, except for the control line which was left unchanged. This parallels SE-HPLC analyses showing that in these conditions, some aggregation also was occurring, producing large glutenin polymers and unextractable residue proteins, except for the control line again. The change concerned glutenin polymers extracted in 0.5% SDS buffer rather than gliadins. The increase of G_N^0 was the largest, in relative values, for the triple null line, which originally had the lowest viscoelasticity and the highest protein extractability. It demonstrated that even a limited glutenin aggregation is able to induce detectable rheological changes.

Without NEMI, glutenin aggregation of all four lines was strongly enhanced by heating at 70°C as was the height of the viscoelastic plateau. All gluten components were involved in aggregation because the recovered amounts of monomeric gliadins were about halved. Nevertheless, even after the thermal treatment, differences were still observed between control and 5+10 lines on the one hand, and 17+18 and triple null lines on the other. Gluten 5+10 left aside, this difference could be explained by the respective contents in residue protein, composed of unextractable prolamin aggregates.

CONCLUSIONS

HMW-GS are indispensable elements that provide viscoelasticity to a gluten network. Subunits differ by their viscoelastic potential, which is expressed through polymers and aggregates of different size distributions. The influence of SS bonds on aggregation and viscoelasticity was delineated by the effect of heating on prolamin association and gluten mechanical properties. The rearrangement of SS cross-links resulted in larger polymers and aggregates and increased viscoelasticity. This did not abolish, however, subunit-related differences. Changes of rheological properties following heating and cooling cycles showed too that connectivity between covalently cross-linked elements of the network is based on hydrogen bonds.

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