

# Studies on Disulfide Bonds in Glutenin

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## ABSTRACT

Cereal Chem. 65(2):95-100

Two attempts to measure the average number of interchain disulfide bonds (SS) per chain in glutenin were made. The first was to let reduced glutenin slowly reoxidize in dilute solution to look for oligomers in samples taken at intervals. It was hoped that intrachain SS would form first because they are stabler and because dilution minimized interchain contacts. The appearance of dimer bands would show when interchain SS were starting to form: the SH groups still unoxidized would then be a measure of interchain SS. Oligomers did not appear as discrete bands, but as marks on the origin and possibly tailing. Oxidation to glutenin was rapid. The tailing that is well known to occur in the electrophoretic patterns of low molecular weight glutenin suggests that there is little preference for specific pairings of subunits, and consequently they are mostly randomly distributed in glutenin molecules. In the other attempt, glutenin was partly reduced, to varying extents, and SH groups were then blocked. Analysis measured the

fraction ( $\tau$ ) of broken SS. The intrinsic viscosity  $[\eta]_0$  was determined and plotted against the fraction of SS broken. These two quantities,  $\tau$  and  $[\eta]_0$ , were calculated from theory, assuming a range of values for the fraction of junctions with two SS in linear glutenin ( $\delta$ ). The theoretical curves were compared with the experimental points by computer, in the hope that  $\delta$  would be the value giving the best fit. The results were not so clear cut as expected, but  $\delta$  was unlikely to be greater than 0.3 and could be zero. It was shown that a previous approach to this problem, also based on viscometry, led to a similar conclusion. This means that if glutenin has linear molecules at least two-thirds, and possibly all, the junctions are likely to consist of a single SS. The exponent,  $\nu$ , in the Mark-Houwink equation ( $[\eta]_0 = KM^\nu$ ) for glutenin appears to be about 0.5. A possible way of distinguishing interchain SS when all subunit sequences are known is suggested.

The polypeptide chains of glutenin, the protein responsible for the viscoelasticity of dough, are believed by most workers to be joined by disulfide bonds (SS). Because each chain has more than one SS, a major unsolved problem is how many SS are interchain. If there is only one per chain, glutenin molecules must be linear concatenations (Ewart 1968). If there are more than one, the molecules are probably branched, but a linear molecule with two SS between neighboring chains is conceivable (Ewart 1972a).

Remarkable progress is being made in finding the amino acid sequences of glutenin chains, and in showing that half-cystine residues are near the ends of high molecular weight subunits (Field et al 1982, Mifflin et al 1983, Forde et al 1983, Moonen et al 1985). Even when all sequences are known, however, it is not easy to see how half-cystine residues of intrachain SS can be distinguished from those of interchain SS. There are very few ways of tackling the problem. Titration with sulfite suggested an average of two interchain SS per chain (Ewart 1972b). The lack of a point of inflection in the graph of viscosity of gluten solutions against time, when a reducing agent was present, was taken to mean that there was only one SS between chains (Ewart 1979), so the second labile SS must be intrachain. It can be shown, however (Appendix A), that though this method can decide unequivocally in favor of one SS bond per chain when the other possibility is two or more, if both single SS and double SS junctions were possible, the presence of up to 30% of double junctions could not be ruled out.

Matsumura et al (1984) blocked sulfhydryl (SH) groups in glutenin after partial reduction and concluded that four major subunits had 0.3 or less interchain SS, which implies that glutenin is not polymeric. They argued that partial reduction catalyzed sequences of interchange reactions to give monomers. But this would mean that the monomeric state is preferred and glutenin is unstable. As they stated later (Kawamura et al 1985), another explanation might be incomplete alkylation when precipitation had occurred. They devised an ingenious approach of reducing glutenin at pH 4.0 and blocking SH groups with iodoacetamide. Excess 2-mercaptoethanol (2-ME) was dialyzed away, and the blocking was finished at pH 8.0 in urea. Subunits of this partly reduced glutenin were separated by electrophoresis, fully reduced, and blocked with vinylpyridine. Six subunits all had about two carboxymethylcysteine (CMC) residues each, i.e., two interchain

bonds, which suggested that glutenin is linear, provided the assumption is true that only interchain SS were reduced in acid. Beckwith and Wall (1966), however, showed that reduced glutenin reoxidized in acid solution at 1 g/L to a low molecular weight product, because interchain SS had reformed as intrachain. (This finding is unlikely to be invalidated if their glutenin contained some gliadin and water-soluble proteins.) Tests here showed that 2-ME can reduce two-thirds the SS at acid pH, so some intrachain SS must have been broken too. Moreover, at acid pH, blocking is so slow that reoxidation competes with it. O<sub>2</sub>-free N<sub>2</sub> has about 5 ppm, v/v, O<sub>2</sub>, and bubbling overnight could supply enough O<sub>2</sub> to oxidize some SH after the 2-ME dialyzed away. This would give a low value for the number of interchain SS in the work of Kawamura et al (1985).

Therefore, it seems doubtful whether Kawamura et al (1985) have established that there is only one interchain SS per chain. (In their calculation they used 5 for the number of SS in each chain of the dimer instead of 2.5; therefore, the average number of intrachain SS per chain of the dimer is 2.05, not 2.3. Thus their second explanation of the nonintegral figures for intrachain SS is unlikely. Sulfur-containing amino acids are difficult to measure accurately, and instrument error could be part of the explanation.)

The studies in this paper seemed to be the only practicable ways of tackling the problem.

## MATERIALS AND METHODS

### Gluten and Glutenin Preparations

Gluten and glutenin were prepared substantially as before (Ewart 1985).

### Reoxidation of Reduced Glutenin

Glutenin was reduced in 8M urea, 20 g/L 2-ME, both with and without 10 g/L of sodium dodecyl sulfate (SDS) under N<sub>2</sub> for 4 hr, then dialyzed in a long dialysis tube in a stirred beaker against water (or 1 g/L of SDS if SDS was used), with two changes a day. Protein concentrations were 20  $\mu$ M, 0.4  $\mu$ M, or 0.13  $\mu$ M, the molarities being based on an average glutenin subunit of mol wt 50,000, which was calculated from data of Huebner and Wall (1974).

To sample, the contents of the tube were mixed, and enough liquor was pinched off into one end; the tube was reknotted, and the sample was cut off. Part of the sample (1 ml) was treated with acrylonitrile (50  $\mu$ l) for 30 min, acidified, freeze-dried, and run on SDS polyacrylamide gel electrophoresis (PAGE).

In later work the reducing solution included 0.35M Tris-HCl,

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pH 8.6, and ethylenediaminetetraacetic acid (EDTA) (2 g/L). Blocking with a slight molar excess of iodoacetamide over the original 2-ME was also tried; it was dissolved in 10 ml of Tris-HCl, pH 8.6, and reacted for 15 min. The solution was acidified with acetic acid, dialyzed in the dark, and freeze-dried.

### SH Determination

Two specimen tubes each had 0.1M Tris-HCl, pH 8, 1 g/L SDS (5 ml). To one was added 1 g/L SDS (1 ml), and to the other the sample (1 ml). Ellman's reagent (0.2 ml of a 10 mM solution in ethanol) was added to each, and the sample was read against the blank at 412 nm.

### Protein Concentration

To correct for change of concentration of the sample during dialysis, the absorbance was read at 280 nm, using 5-cm cells for dilute solutions.

### Absorption Coefficient

Glutenin solutions of known concentration and solvent were weighed into a spectrophotometric cuvette and read at 280 nm. Haze corrections were applied by extrapolating a baseline from higher wavelengths after recording the spectrum up to 400 nm. Absorbance =  $C \times 1.21 \pm 0.05$  (standard error of the mean) where  $C$  is in grams per liter.

### Partial Reduction of Glutenin

O<sub>2</sub>-free N<sub>2</sub> was bubbled through water (1 L) for 30 min, and the appropriate volume of 2-ME added (the final protein/ME ratio ranged from 540 to 22). This solution (1 ml) was added to glutenin (0.7 g protein) suspended in 0.35M Tris-HCl, pH 8.6, EDTA (2 g/L; 20 ml) and stirred for 15 min. Iodoacetamide (2:1 molar ratio to 2-ME) in the buffer (1 ml) was added at once. After stirring for 15 min at room temperature (~20°C), the mixture was acidified, dialyzed against ~0.01M acetic acid, and freeze-dried. The sample corresponding to point (0.17, 0.94) in Figure 1 was reduced in ice.

### Intrinsic Viscosity Measurements

The solvent was A.R. urea (72 g), acetic acid (4.5 ml), and SDS (7.5 g) in water (80 ml). Measuring the intrinsic viscosity of native glutenin was done as described (Ewart 1980). Reduced glutenin (0.3–0.4 g) was weighed into a 16-ml plastic ultracentrifuge tube,

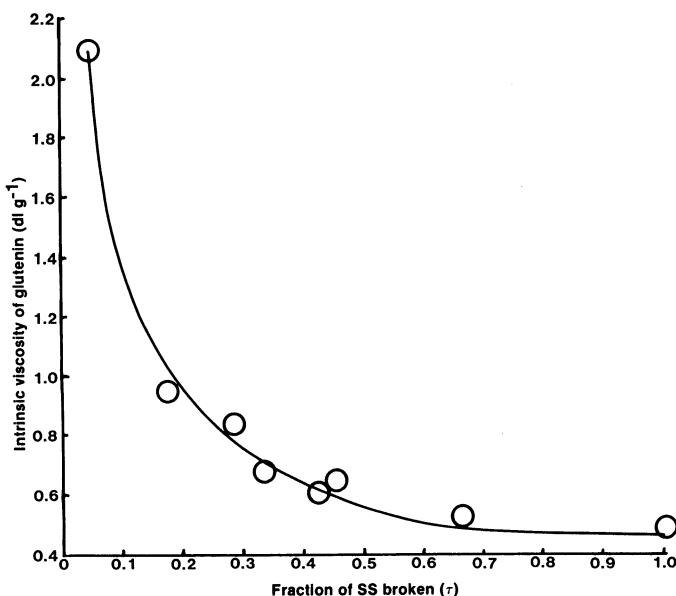


Fig. 1. Theoretical curve of the relation between intrinsic viscosity of glutenin and fraction of total disulfide bonds (SS) broken for "2 carboxymethylcysteine,"  $\nu = 0.48$ ,  $k_i/k_r = 0.18$ ,  $\delta = 0$ . Circles mark experimental points.

and solvent (10 ml) was added. The mixture was vigorously stirred with a magnet overnight and centrifuged at  $59,000 \times g$  for 15 min. The supernatant layer was filtered through a tuft of glass wool in a small funnel and used for intrinsic viscosity measurements as before (Ewart 1980). The pellet and glass wool were mixed in the tube filled with solvent and re-centrifuged for 30 min. Both a known volume (~1 ml) of supernatant layer and the washed pellet plus glass wool were separately dialyzed against water, freeze-dried, and hydrolyzed with 6M HCl at 105°C for 23 hr in screw-cap tubes fitted with Teflon washers. The pipette was calibrated for each solution. Residual urea after dialysis would cause serious errors if Kjeldahl N were measured, but amino acid analysis enabled the amount dissolved to be calculated so that the protein concentrations, ultimately based on  $5.7 \times$  Kjeldahl N, could be found. The average molecular weight of an anhydro amino acid in glutenin was taken as 110. Because the volume of the ~1-ml sample could not be determined accurately without the density of the glutenin solution, successive approximations had to be made. The amino acid loss in hydrolysis was assumed to be the same for the pellet sample and the solution sample; it could then be calculated by comparing the original protein estimated from amino acid analyses with the actual value in the weighed sample of glutenin.

### Amino Acid Analysis

The samples were hydrolyzed with 6M HCl in sealed evacuated (<0.05 mm Hg) tubes at 105°C for 23 hr, rotary evaporated at <40°C, taken up in LKB loading buffer, pH 2.2, spin-filtered (0.2  $\mu$ m), and analyzed for amino acids including CMC.

### Rate Constants

Approximate net rate constants were calculated for the reaction of 2-ME with SS bonds in glutenin by solving the standard equation for a second-order reaction. The CMC value, together with half an occasional small amount of cysteic acid, directly measured broken SS. The original SS level was half the total (half-cystine +  $2 \times$  CMC + any cysteic acid).

### Electrophoresis

Slab 5% PAGE in the presence of SDS was done substantially as described by Laemmli and Favre (1973) except that 2-ME was excluded.

## RESULTS AND DISCUSSION

### First Try at Measuring Interchain SS

Oxidation was done near physiological pH, in the hope that the stabler SS (i.e., the intrachain, from the work of Cecil and Wake [1962]) would form first because they would be likely to lie in a deeper potential energy well than the interchain would, and the dilute solution would make interchain bond formation slow because of the infrequent collisions between chains. The formation of interchain SS would be signalled by dimer and trimer bands appearing on SDS-PAGE without 2-ME (5% gels were used to spread the bands out better). At this point the average number of SH groups left per chain would show how many half-cystine residues per chain formed interchain SS.

Several attempts, with or without SDS, were made. Later, urea was dialyzed off in the presence of 2-ME in case it prevented native conformations being reached. Oxidation of SH groups was rapid once the 2-ME had gone, giving tailing and marks at the origin instead of bands. The tailing, however, may have been due, in part at least, to the hydrophobic components of glutenin, probably membrane proteins, that were found in the A fraction of Huebner and Wall (1974) and Huebner et al (1974).

Wetlaufer and Saxena (1970) found that the concentration of reduced proteins should not be more than 1  $\mu$ M during reoxidation, otherwise insoluble material formed. In case the concentration of 1 g/L (20  $\mu$ M) used by Beckwith and Wall (1966) was too high near neutral pH, it was lowered as far as 0.13  $\mu$ M, but glutenin still formed without appreciable amounts of discrete bands. It is not known whether structures similar to that of native glutenin were formed or not. Beckwith and Wall (1966) were not

quite able to recover the properties of native glutenin after reoxidation.

### Possible Random Order of Chains in Glutenin

If the order of chains in glutenin molecules were random, then initial reoxidation should form all possible dimers and higher oligomers. The number of *i*-mers is easily calculated (Appendix B).

Taking  $n = 12$  for the average SDS-PAGE pattern of glutenin gives 78 dimers, 364 trimers, and 1,365 tetramers, etc.

Therefore, if there were no preference for particular oligomers, the resulting bands, with an average width of say 1.5 mm, could fill the space available several times over, leading to tailing. Tailing is well known to occur when lower molecular weight glutenin is run on SDS-PAGE without 2-ME or ordinary gel electrophoresis and suggests that the arrangement of chains in glutenin is random.

Lawrence and Payne (1983) were the first to discover oligomers (probably dimers) of some high molecular weight subunits of glutenin. Similar bands have since been detected here in glutenin, where one-eighth to two-thirds the SS were broken, but they have not been studied further. Lawrence and Payne (1983) concluded that either some subunits combined preferentially with one another, or some interchain SS were unusually stable. Their work showed that a 10× increase in 2-ME has little effect on the dimers but markedly raises the "specific" subunits to their levels in the fully reduced sample. Presumably then, most of the specific subunits are combined with other subunits, and only a minority combine among themselves. The amounts of these dimers are too small, on present evidence, to support a serious departure from randomness in the distribution of subunits in glutenin molecules.

To study the formation of glutenin, it would be better to test only one or two chains at a time.

### Second Try at Measuring Interchain SS

Glutenin samples were partly reduced and the SH groups were blocked with iodoacetamide. The ratios of protein to 2-ME were higher than or in the range covered by solvents 2 and 3 of Lawrence and Payne (1983), but reaction was at a lower temperature, for a much shorter time, and in the absence of SDS. The decrease in solubility as the fraction of total SS broken ( $\tau$ ) falls (Table I) resulted from larger molecules dissolving more slowly when only magnetic stirring was used. For this reason, each ultracentrifuged solution was analyzed separately for intrinsic viscosity of glutenin  $[\eta]_0$  and  $\tau$ . It was hoped that  $[\eta]_0$  would fall to a minimum if all the interchain SS reacted before the intrachain. Experiment disproved this, because a curve resembling that of exponential decay was obtained (Fig. 1). Evidently intrachain SS were broken concurrently with interchain. The value of 0.48 dl/g for fully reduced and blocked glutenin is in line with 0.295 for reduced and blocked A gliadin, mol wt 31,900 (Cole et al 1984), because the glutenin chains are bigger.

For linear glutenin having two SS in some of or all the junctions, a relation can be calculated (Appendix C) between  $[\eta]_0$ ,  $\delta$  (the fraction of junctions having two SS), and  $\tau$  (the fraction of total SS

broken). T. Fearn, a colleague, devised a computer program to evaluate the expressions, plot the experimental and theoretical points, and calculate the total squared deviations in  $[\eta]_0$ . This program showed how good the fits were, because the smaller the total squared deviations, the better the fit (Table II). The best fit is plotted in Figure 1; none of its points deviate significantly from the theoretical curve, because all lie within the experimental error (i.e., twice the standard error of the mean, calculated from the replication).

### Calculation of SS Broken

At low concentration, 2-ME would produce residues of mixed disulfide and cysteine when it attacked an SS. After hydrolysis the mixed disulfide between cysteine and 2-ME (CysME) would appear on the trailing edge of proline. Because peaks corresponding to CysME were seen,  $\tau$  was based on twice the CMC level (except in the case of full reduction). That this was a more accurate way of measuring  $\tau$  was clear when values based on 1× CMC were tried (Table II); fits were poor at all values of  $\delta$ .

### Exponent in Mark-Houwink Equation

The Mark-Houwink equation ( $[\eta]_0 = KM^\nu$ ) enables  $M$ , the average molecular weight of the glutenin, to be calculated from the intrinsic viscosity. A factor of 4 rather than 2 in the formula for calculating the average number of chains per glutenin molecule (Ewart 1985) led to better agreement with the limited molecular weight data in the literature. The factor 4 (which is the average number of small thiols bound per glutenin molecule) corresponded to  $\nu = 0.48$  in the Mark-Houwink equation (Appendix C), which is at the bottom end of the range, 0.5 to 0.8 (Flory 1953), allowing for experimental error. The factor 2 gave  $\nu = 0.63$ . Taking 0.48 for  $\nu$  gave a much better fit between experiment and theory than taking 0.63 (Table II).

### Values for $\delta$

The results are compatible with a value of zero for  $\delta$ , but Fearn showed that values from 0 to 0.3 lie within the 95% confidence limits, which agrees with the finding from the previous approach (Ewart 1979) (Appendix A).

This study has appreciably narrowed the limits to the number of junctions in linear glutenin that may have two SS. The problem of finding the true value of  $\delta$  appears insoluble, at present.

The good fit suggests that the calculated molecular weight distribution for glutenin (Ewart 1987) is at least near the true one.

### Possible Strategy for Identifying Interchain SS

It may be possible to bridge interchain SS with a fissile cross-linker, after which the SS could be reduced and blocked, the glutenin digested with an enzyme, and the peptides containing cross-links isolated. When the cross-link was broken, interchain SS would give two peptides. Intrachain would give only one, provided that the peptide chain between the cross-links had not been cut when glutenin was digested. Once the amino acid sequences in the cysteine-containing regions are all known, amino acid analysis of an oligopeptide should identify it. By sequence matching, it should be possible to tell whether or not the two fragments of peptide chain were contiguous in a subunit. If they

TABLE I

Intrinsic Viscosity of Glutenin at Various Extents of Disulfide (SS) Fission

$[\eta]_0$ (dl/g)	Fraction of Total SS Broken ( $\tau$ )	% of Sample Dissolved
2.08 (4) <sup>a</sup>	0.04	... <sup>b</sup>
0.94 (3)	0.17	83
0.83 (3)	0.28	88
0.67 (3)	0.33	92
0.64 (3)	0.45	91
0.60 (3)	0.42	98
0.52 (3)	0.66	98
0.48 (4)	1.00	99

<sup>a</sup>Number of determinations in parentheses.

<sup>b</sup>Determined from measurements on gluten, when the solubility was above 99%.

TABLE II

Minimum Sum of Squared Errors ( $\times 10^3$ ) Between Theory and Experiment for  $[\eta]_0$  at the Eight Values of  $\tau$

$\delta$	$\nu = 0.48$		$\nu = 0.63$	
	2CMC <sup>a</sup>	CMC	2CMC	CMC
0	15	125	222	85
0.05	17	123	238	91
0.1	19	122	255	97
0.2	26	117	296	112
0.4	54	109	409	154
1.0	1,010	459	1,852	1,123

<sup>a</sup>CMC = carboxymethylcysteine.

were contiguous, the SS was probably (though not certainly) intrachain.

#### ACKNOWLEDGMENTS

This work forms part of a project sponsored by the U.K. Ministry of Agriculture, Fisheries, and Food (MAFF), to whom thanks are due. The results of the research are the property of the MAFF and are ©British Crown copyright 1988. The author thanks Dr. T. Fearn for divising a way of testing for goodness of fit and for all the computer calculations, and Mr. D. Smith for experimental assistance.

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#### APPENDIX A

##### Inflection Point During Glutenin Degradation

If the number-average degrees of polymerization (DP) of glutenin are P<sub>0</sub>, P<sub>t</sub> at times 0 and t, respectively (Ewart 1968)

$$P_t = \frac{P_0}{1 + (P_0 - 1)b} \quad (1)$$

where *b* (formerly  $\alpha$ ) is the chance an interchain junction is broken. The equation is independent of the molecular weight distribution. A fraction  $\delta$  of the junctions have double SS bonds. If  $\phi$  is the chance that any interchain SS is broken, then

$$b \text{ is } (\text{chance that a junction is double}) \times (\text{chance that it is broken}) \\ + (\text{chance that a junction is single}) \times (\text{chance that it is broken}) \\ = \delta \phi^2 + (1 - \delta)\phi \quad (2)$$

Hence

$$P_t = \frac{P_0}{1 + (P_0 - 1)(\delta \phi^2 + (1 - \delta)\phi)} \quad (3)$$

The graph of P<sub>t</sub> against  $\phi$  will have an inflection point when  $d^2P_t/d\phi^2 = 0$ . (At a point of inflection the slope  $dP_t/d\phi$  has a maximum or minimum,  $\therefore$  its derivative, i.e.  $d^2P_t/d\phi^2 = 0$ .)

$$\frac{d^2P_t}{d\phi^2} = \frac{2P_0(P_0 - 1)[(P_0 - 1)(1 - \delta + 2\delta\phi)^2 - \delta(1 + (P_0 - 1)(\delta\phi^2 + (1 - \delta)\phi))]}{[1 + (P_0 - 1)(\delta\phi^2 + (1 - \delta)\phi)]^3} \quad (4)$$

Putting the right hand side = 0 leads to a quadratic equation in giving the condition for a point of inflection:

$$\phi^2 + [(1 - \delta)/\delta]\phi + 1/3[(1 - \delta)/\delta]^2 - 1/[3\delta(P_0 - 1)] = 0 \quad (5)$$

whence

$$\phi = 1/2 - 1/2\delta + \sqrt{1/[3\delta(P_0 - 1)] - (1 - \delta)^2/12\delta^2}$$

( $\delta$  is a fraction, so  $1/2 - 1/2\delta$  is negative.  $\therefore$  since  $\phi$  must be positive, only the positive value of the square root need be considered).

$$\therefore \sqrt{1/[3\delta(P_0 - 1)] - (1 - \delta)^2/12\delta^2} > -(1/2 - 1/2\delta)$$

whence

$$P_0 < 1 + \delta/(1 - \delta)^2 \quad (6)$$

To calculate P<sub>0</sub> for gluten rather than glutenin, the mean molecular weights of chains were taken as 35,000 and 50,000, and the fractions as 0.525 and 0.475, for gliadin and glutenin, respectively.

Taking a mass of *wg* of gluten, and *L* as the Avogadro number, there will be 0.525 *wL*/35,000 gliadin molecules and 0.475 *wL*/50,000 P<sub>u</sub> glutenin molecules, where P<sub>u</sub> is the average number of chains in a glutenin molecule. As is shown later, this was taken as 25.

The number of gliadin chains is the same as the number of gliadin molecules. Number of glutenin chains = P<sub>u</sub> × (no. of molecules).

$$\therefore P_0 = (\text{no. of chains})/(\text{no. of molecules}) \\ = \frac{(0.525 \text{ wL})/(35,000) + (0.475 \text{ wL})/(50,000)}{(0.525 \text{ wL})/(35,000) + (0.475 \text{ wL})/(50,000 P_u)}$$

Putting  $P_u = 25$ ,  $P_0 = 1.59$ .

Whence the condition becomes:  $\delta^2 - 3.695\delta + 1 < 0$ . The LHS is only  $< 0$ , i.e., negative, when  $\delta$  lies between 0.294 and 3.401. Therefore the condition for a point of inflection to appear is when  $\delta$  lies between 0.3 and 1.0 since  $\delta$  cannot  $> 1$ . Hence in the previous work (Ewart 1979), if up to 30% of the junctions in glutenin had two SS, this would have escaped detection. (On p. 490 of Ewart [1979], the phrase "(values of  $x$  above unity)" is wrong and should be deleted.)

## APPENDIX B

### Number of Arrangements of $n$ Different Things

If  $A$  is the number of ways  $n$  different letters, say, can be arranged in a row, the arrangements can be classed by their first letter in  $n$  equal classes. Each class starting with the same letter can be subdivided according to the second letter into  $n - 1$  equal sets (since one letter is used up for the first place there are  $[n - 1]$  left to choose the second letter from). Each set can be split into  $(n - 2)$  equal groups, the members of a given group having the same sequence for their first three letters; with the first two letters in a set determined, the number of choices for the third letter in a group is now  $(n - 2)$ . Groups can be further subdivided, the number of choices falling by one at each division until the  $A$  arrangements have been subdivided into single arrangements when there is only one letter left to fill the last place. Thus  $A$ , the number of arrangements, must be  $n \times (n - 1) \times (n - 2) \times \dots \times 4 \times 3 \times 2 \times 1$ . This is written  $n!$

### Number of Ways of Arranging Two Groups of Identical Objects

Let  $Q$  be the number of ways  $B$  black balls and  $W$  white balls can be arranged in a row. Consider any one of these arrangements, and now number the black balls. Instead of one arrangement, the now distinguishable black balls can be distributed in  $B!$  ways among the places they occupy in that particular arrangement. Therefore, with numbered black balls there  $Q \times B!$  possible arrangements altogether. Similarly if the white balls are now numbered, the total number of arrangements becomes  $Q \times B! \times W!$ . But since the balls are now all distinguishable, the number of arrangements must also be  $(B + W)!$

$$\therefore Q = (B + W)! / B! \times W! \quad (7)$$

### Number of Glutenin i-mers, Ignoring Order

Let  $B = n - 1$  and  $W = i$ , then  $Q = (n + i - 1)! / i! (n - 1)!$ . In every arrangement the  $(n - 1)$  black balls can be regarded as partitions; with the  $(n - 2)$  gaps between them and the two outside positions they divide it into  $n$  boxes. The number of glutenin i-mers, assuming that the order in which the chains are combined does not affect the mobility on SDS-PAGE, is the number of ways choosing  $i$  chains from the  $n$  that are distinguishable on SDS-PAGE. Choosing a chain can be symbolized by putting a white ball in the box corresponding to that particular chain.  $(n + i - 1)! / i! (n - 1)!$  is the number of possible ways in which the  $i$  white balls can be distributed in  $n$  labelled boxes, when empty boxes are allowed. (In arrangements where, say, the fifth and sixth black balls from the left were touching, the sixth box from the left would be empty—remember that the first ball is the right-hand wall of the first box.) Thus, this formula must be the number of ways of making up glutenin i-mers from  $n$  chains, where the order of the chains in an i-mer is ignored.

## APPENDIX C

### Fraction of Disulfide Bonds Broken

$N$  polypeptide chains, of various kinds, are assumed to be distributed at random in glutenin molecules. The average number of SS per chain is  $s$ , including intrachain.

The average number of labile, or easily reduced, SS per chain is  $\lambda$ ; these are the interchain SS and the labile intrachain SS.

There are thus  $N\lambda$  labile SS and  $N(s - \lambda)$  stable SS.

The native glutenin may be regarded as a very large molecule that has been degraded by random attack of small thiols, i.e., endblockers (EB) (Ewart 1985). Reaction of EB with stable intrachain SS is likely to be negligible, if there is any. A fraction,  $\phi_n$ , of labile SS are broken by EB in native glutenin. Thus, the number of SS broken in native glutenin is  $N\lambda\phi_n$ .

During partial reduction with 2-ME, the fraction of labile SS broken increases to  $\phi$ .

Thus, the number of labile SS broken =  $N\lambda\phi$ .

The fraction of stable SS reduced by 2-ME is  $\sigma$ . Therefore, number of stable SS broken =  $N(s - \lambda)\sigma$ .

The experimentally observed quantity,  $\tau$ , the fraction of total SS broken,

$$= [N\lambda\phi + N(s - \lambda)\sigma] / Ns$$

$$\therefore \tau = [\lambda\phi + (s - \lambda)\sigma] / s. \quad (8)$$

In this treatment, the contributions of EB towards the total SS, and the stable interchain SS (Lawrence and Payne 1983), are ignored because they are less than the error of amino acid analysis, and those EB that are reduced would be lost by dialysis after the blocking stage.

### Estimation of $\sigma$

The net reaction of 2-ME with SS bonds will be governed by two equations:

$$-(dC_s/dt) = k_s C_m^q C_s \quad (9)$$

and

$$-(dC_f/dt) = k_f C_m^q C_f \quad (10)$$

where  $C$  is the concentration, subscripts  $s$ ,  $f$ , or  $m$  referring to slow SS, fast SS, or 2-ME, respectively, and  $q$  is 1 or 2 depending on whether there is enough 2-ME to react with one or both halves of cystine.

Dividing (9) by (10) leads to:

$$dC_s/dC_f = (k_s C_s) / (k_f C_f) \quad (11)$$

Integrating:

$$\ln C_s = (k_s/k_f) \ln C_f + Z \quad (12)$$

where  $Z$  is a constant.

When  $t = 0$ ,

$$\ln C_{s0} = (k_s/k_f) \ln C_{f0} + Z$$

where the subscript 0 stands for the concentrations at the start of the reaction with 2-ME

$$\therefore \ln (C_s/C_{s0}) = (k_s/k_f) \ln (C_f/C_{f0}) \quad (13)$$

But  $\sigma = 1 - (C_s/C_{s0})$ ,

and

$$\begin{aligned} (C_f/C_{f0}) &= \frac{\text{no. of labile SS left after reduction}}{\text{no. of labile SS at start of reduction}} \\ &= \frac{N\lambda - N\lambda\phi}{N\lambda - N\lambda\phi_n} \end{aligned}$$

$$= \frac{1 - \phi}{1 - \phi_n} \quad (14)$$

∴ (13) becomes:  $\ln(1 - \sigma) = (k_s/k_r)\ln[(1 - \phi)/(1 - \phi_n)]$

$$\therefore \sigma = 1 - [(1 - \phi)/(1 - \phi_n)]^{k_s/k_r} \quad (15)$$

and

$$\tau = \frac{\lambda\phi + (s - \lambda) \left\{ 1 - [(1 - \phi)/(1 - \phi_n)]^{(k_s/k_r)} \right\}}{s} \quad (16)$$

### Intrinsic Viscosity

The other experimentally observed quantity is intrinsic viscosity of glutenin,  $[\eta]_u$ . This can be written, after slight modification (Tanford 1961),

$$[\eta]_u = \frac{K \sum_{i=1}^{\infty} n_i M_i^{\nu+1}}{\sum_{i=1}^{\infty} n_i M_i} \quad (17)$$

where  $K$  and  $\nu$  are constants,  $n_i$  is the number of molecules with  $i$  polypeptide chains, and  $M_i$  is the molecular weight of an  $i$ -mer. If  $m$  is the average molecular weight of a glutenin subunit,

$$[\eta]_u = \frac{K \sum_{i=1}^{\infty} n_i (mi)^{\nu+1}}{\sum_{i=1}^{\infty} n_i mi} \quad (18)$$

It is necessary to know the molecular weight distribution to evaluate this. Substituting for  $n_i = Nb^2(1 - b)^{i-1}$  from the calculated molecular weight distribution for glutenin (Ewart 1987),

$$[\eta]_u = \frac{K \sum_{i=1}^{\infty} Nb^2(1 - b)^{i-1} (mi)^{\nu+1}}{\sum_{i=1}^{\infty} Nb^2(1 - b)^{i-1} mi} \quad (19)$$

The  $N$  and  $m$  cancel and the denominator is unity.

$$\therefore [\eta]_u = K \sum_{i=1}^{\infty} b^2(1 - b)^{i-1} m^{\nu} i^{\nu+1} \quad (20)$$

Substituting from equation (2) for  $b$  gives:

$$[\eta]_u = Km^{\nu} \sum_{i=1}^{\infty} (\delta\phi^2 + (1 - \delta)\phi)^2 [1 - (\delta\phi^2 + (1 - \delta)\phi)]^{i-1} i^{\nu+1} \quad (21)$$

The computer calculated from equation (16) the values of  $\phi$  corresponding to the eight experimental values of  $\tau$ , and then calculated  $[\eta]_u$  from equation (21). It chose the constant  $K$  (which merely affected the position of the graph on the vertical axis) to give the closest fit to the eight experimental points in each case.

### Values Used

The value for  $\lambda$  was 2 determined from earlier work (Ewart

1972b). Lawrence and Payne (1984) have confirmed this for high molecular weight glutenin subunits with a figure of four active -Cys.

The mean value of  $s = 4.0$  came from experimental values on the Canada western red spring native glutenin, determined under similar conditions to those used for the reduced glutenins and with the same analyzer.

### $k_s/k_r$

The exact mathematical treatment of concurrent reactions is difficult. (An attempt in a textbook [Moelwyn-Hughes 1961] to treat a simple case is in error because all the halide used is assumed to have reacted with hydroxyl ion, whereas some must have reacted with water.)

In the early stage of reduction the rate is governed mainly by the interchain SS, and in later stages, when most of the interchain have reacted, by the intrachain. In both cases the rate constants found this way may be somewhat larger than the true constants because the amount reacted would be increased by the concurrent reaction, though this would be at least partly offset by the reversibility of the reaction. These errors would tend to cancel, when the ratio of the constants is taken. Calculation of the true rate constants of the forward stage of a reversible reaction should only be done when the back reaction has not had time to become significant. In this work, however, only the rate constants of the *net* forward reactions are of interest, so it did not matter if the reverse reaction was significant and probably faster for the stable SS. In any case,  $\tau$  was not greatly affected by variations in  $k_s/k_r$  because  $\sigma$  is fairly small compared with  $\phi$ .

The values for  $k_s$  and  $k_r$ , each the means of seven and five determinations, respectively, together with their standard errors were  $0.14 \pm 0.02$  and  $0.77 \pm 0.18 \text{ l mol}^{-1}\text{s}^{-1}$ , giving a  $k_s/k_r$  ratio of 0.18.

The point (0.17, 0.94) in Figure 1 corresponds to a sample reduced in ice instead of at room temperature. The Arrhenius equation,  $k = A e^{-E/RT}$ , shows that the  $k_s/k_r$  ratio is likely to drop to about 0.15 at  $0^\circ\text{C}$ . This would cause only a 1.3% drop in the calculated value of  $[\eta]_u$  at that point bringing it nearer the experimental point. This justifies retaining this point in Figure 1.

### $\nu$

The value for  $\nu$  was obtained by fitting  $\log_{10} [\eta]_u$  for six wheats to the  $\log_{10}$  (mol wt) of the glutenins where the molecular weights were calculated from measurements of small thiols bound to glutenin by SS, this being the only known way of estimating them (Ewart 1985). The equation was  $\log_{10} [\eta]_u = 0.478 \log_{10} (\text{mol wt}) - 2.566$ , whence  $[\eta]_u = 2.7 \times 10^{-3} \times (\text{mol wt})^{0.48}$ .

### $\phi_n$

Because the experimentally measured quantity, 0.16, for the moles of EB per mole of chain in the native glutenin is also given by:

$$\frac{2\lambda N\phi_n}{N} = 2\lambda\phi_n$$

a value for  $\phi_n$  of 0.04 was calculated. This leads to an average figure of 25 chains for a Canada western red spring glutenin molecule ( $P_u$ ).

[Received March 18, 1987. Revision received July 23, 1987. Accepted August 6, 1987.]