Apolar Interactions of α-Gliadin: Binding of 2-p-Toluidinylnaphthalene-6-Sulfonate

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ABSTRACT

The interactions of the wheat prolamine α-gliadin with 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) have been studied by fluorescence spectrophotometry and equilibrium dialysis. The fluorescence of TNS is enhanced in the presence of α-gliadin and the fluorescence yield of the protein•TNS couple is directly related to the extent of TNS binding as determined by equilibrium dialysis. Alpha-gliadin can bind appreciable amounts of TNS. The binding is interpreted as evidence of accessible apolar regions on the surface of the protein molecule. TNS binding is stronger at pH 3.1 than pH 5.0. This pH sensitivity may be related to conformational change in α-gliadin. An increase in the degree of protein aggregation may accompany the binding of TNS, probably as a result of partial neutralization of positive charges on the protein.

The important contributions of electrostatic and hydrogen-bonding forces to the properties of gliadin, the prolamine fraction from wheat endosperm, have been documented by Holme and Briggs (1) and by Beckwith et al. (2). The interplay of these forces is apparent in the pH and ionic-strength dependence of the aggregation behavior of gliadin proteins, and helps determine the contribution of gliadin to dough properties. The role of apolar (hydrophobic) interactions in determining the properties of gliadins has not yet been clearly assessed. The high proportion of apolar amino acid side chains characteristic of this class of proteins suggests, however, that some aspects of gliadin behavior will be controlled or influenced by apolar interactions. Potential areas of influence include conformational stability, aggregation behavior, and binding of lipids. The present study is designed to aid evaluation of apolar contributions to gliadin properties by determination of the nature and spatial extent of the molecule's accessible apolar regions. Some insight may thus be gained into the potential for intermolecular apolar interactions.

For this purpose, α-gliadin prepared by the procedure of Bernardin et al. (3) is considered, by virtue of similarities in molecular weight, amino acid composition, and lipid-binding potential[2], as representative of gliadin proteins. The experimental approach utilizes the properties of 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS), a "hydrophobic probe", whose fluorescence yield is inversely proportional to the dielectric constant of its immediate environment (4). In aqueous solution with proteins, such an enhancement of TNS fluorescence yield may be interpreted as

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[2]When acetic acid extracts of several wheat flours were subjected to polyacrylamide-gel electrophoresis and exposed to a TNS-type hydrophobic probe, all proteins of the gliadin group enhanced the probe's fluorescence (unpublished data).

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evidence that TNS molecules have become associated with accessible apolar regions of the proteins. In the presence of α-gliadin, the fluorescence of TNS is enhanced relative to its value in aqueous salt solution, indicating the presence of apolar regions on the protein's surface. This fluorescence-probe technique has been used, in conjunction with equilibrium dialysis binding studies, to estimate the extent of such regions and their affinity for TNS.

MATERIALS AND METHODS

The flour used was a commercially milled 1968 hard red winter wheat flour, 95% baker's patent, unbleached and unmalted. Alpha-gliadin was prepared from this flour by the method of Bernardin et al. (3). (A clear 0.01M acetic acid extract was centrifuged at 133,000 $\times$ g. The resulting pellet of crude α-gliadin was redissolved and further purified by additional ultracentrifugation and Sephadex gel filtration.) The purified α-gliadin migrated as a doublet in acrylamide-gel electrophoresis (pH 3.1, aluminum lactate buffer (3)).

The potassium salt of 2-*p*-toluidinylnaphthalene-6-sulfonic acid, lot 49B-5030, was obtained from Sigma Chemical Company. Quinine sulfate Merck was recrystallized twice from deionized water. All other materials were commercially available reagent grade.

Fluorescence determinations were made at 20 ± 1°C. with a Turner Model 210 spectrophotofluorometer (G. K. Turner Associates, Palo Alto, Calif.). TNS fluorescence was excited with 347 nm. radiation (100 A bandwidth) and monitored at 445 nm. To achieve desired TNS concentrations in α-gliadin titration experiments, measured amounts of 3×10^{-3}M TNS solution were added to protein solutions with a microsyringe. The total solution absorbance at the exciting wavelength was $\lesssim 0.09$. Quantum yield determinations were based on the method of Parker and Rees (5).

For dialysis experiments, visking tubing was treated as outlined by Rosenberg and Klotz (6). The experiments were done in Plexiglas cells: single-layer membranes from the treated dialysis tubing were clamped between the two halves of a cell, forming chambers of about 1.0- and 2.8-ml. volumes, respectively. Stirring was done either magnetically or by trapped air bubbles in cells rotated about a vertical axis. TNS was added only to the buffer compartment of the cells. Twenty-four to forty hours were allowed for attainment of dialysis equilibrium at a room temperature of 25°C. (7).

Equilibrium TNS concentrations were determined from contents of the protein-free cell chamber by absorbance measurements at 317 nm. The difference in TNS concentration between the two chambers of the cell at equilibrium is due to TNS binding by the protein in one chamber, and is used to estimate the number of TNS molecules bound. Binding of TNS by the membrane and cell components was negligible. In a typical control equipment at pH 5.0, where an equilibrium TNS concentration of 3.11×10^{-4}M was expected in the cell, the concentration observed was 3.09×10^{-4}M. In an experiment at pH 3.1, where 2.50×10^{-4}M was expected, 2.45×10^{-4}M was observed.

Alpha-gliadin stock solutions were prepared in 0.001M HCl-0.004M KCl, pH 3.1, μ (ionic strength) $\simeq 0.005$. Alpha-gliadin molar concentrations are based on a molecular weight of 31,000, as determined by Platt and Kasarda (unpublished

data). The pH was controlled in the equilibrium dialysis experiments by preliminary dialysis of α-gliadin against HCl-KCl solutions of the desired pH (μ = 0.005), with titration if necessary.

RESULTS

Fluorescence Studies

Figure 1A illustrates the effect of α-gliadin on the fluorescence of TNS excited at 347 nm. The curves are proportional to the true emission spectra. The fluorescence of TNS dissolved in pH 5.0 deionized water is clearly enhanced by the presence of α-gliadin (curve 1). Addition of HCl to 0.001M (pH 3.1) caused a large increase in the fluorescence enhancement (curve 2). Subsequent addition of KCl to 0.005M at pH 3.1 caused approximately a 35% decrease in the fluorescence (curve 3); and a further decrease, to approximately one-third of the 0.001M HCl value, occurred upon addition of KCl to 0.05M (curve 4). The fluorescence tendencies of TNS are invariant over the range of pH and ionic strength conditions used in this study (4), so the fluorescence changes observed must be related to changes in the behavior of α-gliadin. In contrast to the KCl concentration effect noted at pH 3.1, no significant difference is apparent at pH 5.0 (Fig. 1B) between the fluorescence of the α-gliadin•TNS couple in the absence of KCl (curve 7) and in the presence of 0.005M KCl (curve 8). The greater sensitivity of α-gliadin•TNS fluorescence to KCl concentration at pH 3.1 than at pH 5.0 suggests that electrostatic factors have a

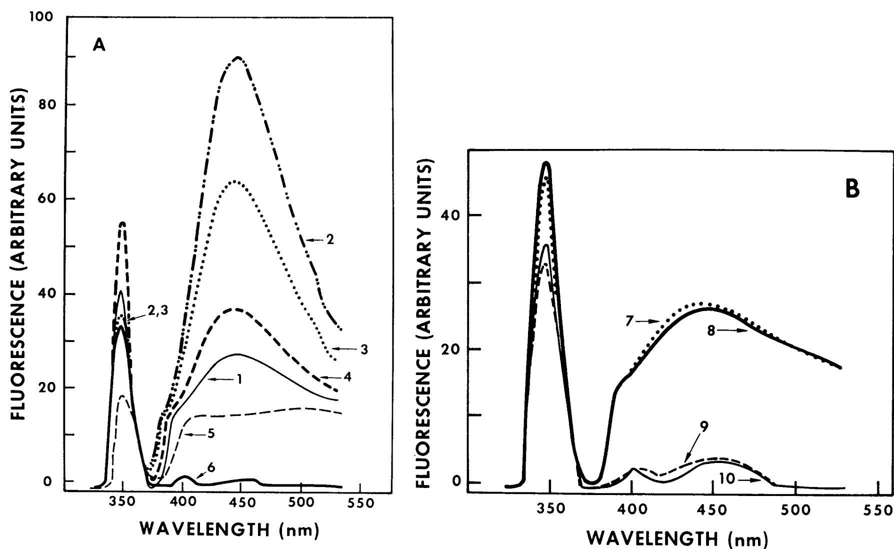


Fig. 1. Effect of KCl concentration on α-gliadin•TNS fluorescence: TNS concentration, 2 × 10^{-5}M; α-gliadin concentration 0.05 mg. per ml. (1.66 × 10^{-6}M). Emission bandwidth 100 A. Peak at 445 nm. is fluorescence; peak at 347 nm. is scattered light.

A: Curve 1, α-gliadin•TNS in pH 5.0 H_2O; Curve 2, in 0.001M HCl (pH 3.1); Curve 3, in 0.001M HCl plus 0.004M KCl (pH 3.1); Curve 4, in 0.001M HCl plus 0.049M KCl (pH 3.1); Curve 5, TNS blank in pH 5.0 H_2O; Curve 6, α-gliadin blank in pH 5.0 H_2O.

B: Curve 7, α-gliadin•TNS in pH 5.0 H_2O; Curve 8, in 0.005M KCl (pH 5.0); Curve 9, α-gliadin blank in pH 5.0 H_2O; Curve 10, α-gliadin blank in 0.005M KCl, pH 5.0.

greater influence on the protein's behavior at the lower pH, probably through ionic screening of the protein's net charge. To simplify data interpretation, subsequent experiments were conducted, if possible, at a total ionic strength of 0.005, which should diminish electrostatic interactions without causing precipitation of the protein. Figure 2 shows results from one such experiment: the α-gliadin•TNS fluorescence is compared to the sum of the α-gliadin and TNS blanks, and enhancement is apparent at both pH 3.1 and 5.0. This allows the conclusion that at pH 5.0, confirming a significant pH effect on the behavior of α-gliadin. Results of a more detailed study of this pH effect are shown in Fig. 3. Here the height of the Rayleigh scatter peak, which is qualitatively proportional to particle size (degree of α-gliadin aggregation), increases with increasing pH; whereas the height of the fluorescence peak, assumed to be proportional to the binding of TNS by α-gliadin, decreases. The pH dependence of the fluorescence is indicative of an α-gliadin transition of some kind, probably affecting its ability to bind TNS. The relation of this to change in degree of aggregation is unclear. The results in Fig. 4 suggest, however, that aggregation is not the sole cause of decreases in fluorescence yield: α-gliadin solutions at pH 9.5 and pH 3.1 (μ = 0.005) have similar scatter peak heights, which indicate low aggregation states relative to that near pH 5.0 to pH

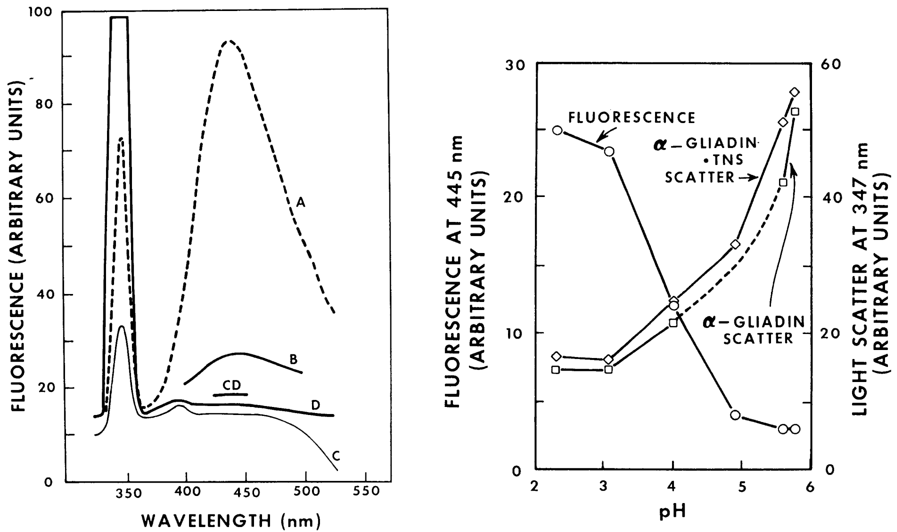


Fig. 2 (left). Effect of pH on α-gliadin•TNS fluorescence at 0.005 ionic strength: TNS concentration 8×10^{-6}M, α-gliadin concentration 0.05 mg. per ml. (1.66×10^{-6}M). Emission bandwidth 100 A. Curve A, α-gliadin•TNS at pH 3.1; Curve B, at pH 5.0; Curve C, pH 5.0, 0.005M KCl; Curve D, α-gliadin blank; Line CD, sum of α-gliadin blank and TNS blank from a separate experiment.

Fig. 3 (right). Effect of pH on α-gliadin•TNS fluorescence and light scattering at 0.005 ionic strength: TNS concentration 2.5×10^{-5}M, α-gliadin concentration 0.05 mg. per ml. (1.66×10^{-6}M); emission bandwidth 25 A; circles = fluorescence; squares = α-gliadin scatter; diamonds = α-gliadin•TNS scatter.

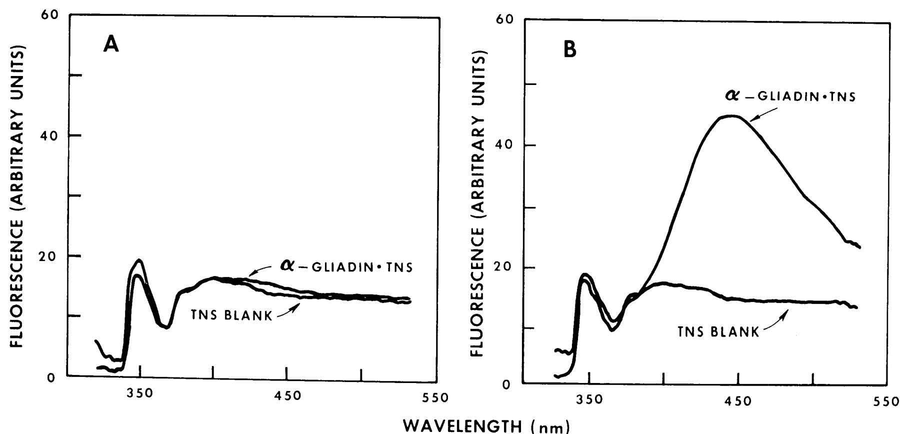


Fig. 4. Fluorescence of α-gliadin•TNS in low-aggregation state at pH 3.1 and 9.5: TNS concentration, 1 X 10^{-5}M; α-gliadin concentration, 0.05 mg. per ml. (1.66 X 10^{-6}M). Emission bandwidth 25 A.

A: 5 mg. per ml. α-gliadin dissolved in pH 3.1, 0.005 μ HCl•KCl, diluted 100 X with pH 9.5, 0.015 μ KOH•KCl, TNS added, and fluorescence curve immediately recorded. TNS blank is pH 9.5, μ = 0.015.

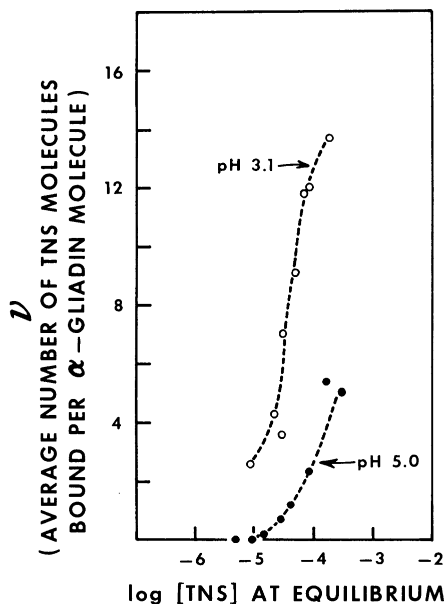
B: TNS added to α-gliadin at pH 3.1, 0.005 μ HCl•KCl. TNS blank is pH 3.1, μ = 0.005.

6.0; yet the α-gliadin•TNS fluorescence is much lower at pH 9.5 than at pH 3.1.

To gain information on the nature of the assumed α-gliadin apolar regions, a determination of the quantum yield of fluorescence of 2.23 X 10^{-7}M TNS in 3 X 10^{-5}M α-gliadin was made. Correction for incomplete α-gliadin•TNS binding was made using binding constants described in the next section. The α-gliadin•TNS fluorescence quantum yield, representative of the highest affinity sites on the α-gliadin molecule, was 0.312 at pH 3.1. This is similar to the value of 0.34 reported (4) for the yield of TNS in bovine serum albumin, a protein demonstrated to have apolar areas with high affinity for hydrocarbon ligands. By analogy, α-gliadin at pH 3.1 may be assumed to have areas of a similar apolar nature.

α-Gliadin•TNS Binding

Equilibrium dialysis experiments were utilized to obtain estimates of the affinity and extent of binding of TNS by α-gliadin. Under the experimental conditions used, the calculated Donnan effect (8, p. 225) would cause a difference by only 1.3 to 2.0% between the TNS concentrations of the two cell chambers, so the binding data are presented uncorrected (Fig. 5). The results are consistent with the qualitative fluorescence observations. It is clear that, at pH 3.1, α-gliadin can bind at least 14 TNS molecules. The average association constant, K_{ass}, estimated from the curve is $\simeq 3 \times 10^4 M^{-1}$. The binding affinity at pH 5.0 seems considerably lower than at pH 3.1. Reciprocal plots of the α-gliadin•TNS binding data are shown in Fig. 6. The Klotz equation $1/\nu = 1/n + 1/nKa$ (9) (where $\nu \equiv$ average number of TNS molecules bound per gliadin molecule at equilibrium TNS concentration [A], and n $\equiv$ maximum number of TNS binding "sites" with affinity constant K available per gliadin molecule) has been used to analyze the data. The pH 3.1 curve



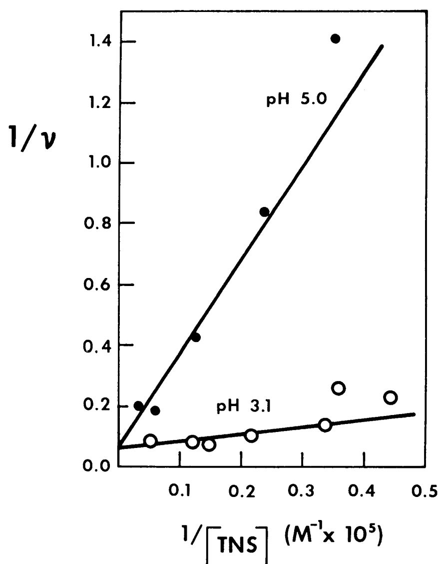


Fig. 5 (left). Binding of TNS by α-gliadin at pH 3.1 and pH 5.0, μ = 0.005: α-gliadin concentration 0.5 mg. per ml. (1.66 X 10^{-5}M). Open circles = pH 3.1; solid circles = pH 5.0.

Fig. 6 (right). Reciprocal plot of α-gliadin•TNS binding data at μ = 0.005; open circles = visual best fit for pH 3.1; closed circles = visual best fit for pH 5.0.

indicates an average binding constant of ∿2.5 X $10^4 M^{-1}$, slightly lower than estimated from Fig. 5. Extrapolation of these points to 1/[TNS] = zero indicates that, in the absence of further perturbation, an α-gliadin molecule may bind a maximum of about 17 TNS molecules at pH 3.1. As each TNS molecule will occupy a surface area of ∿32 square angstroms,[3] compared to a calculated minimum surface area of ∿5,600 square angstroms for a smooth protein sphere of the weight of α-gliadin, this indicates that exposed apolar regions comprise approximately 10% of the protein's surface. The plot from pH 5.0 α-gliadin•TNS data yields a binding constant of 1.8 X $10^3 M^{-1}$, and a probable maximum binding capacity near 17 moles TNS per mole of α-gliadin. The precision of this extrapolation is limited, however, because the protein was less than 50% saturated with TNS at the highest TNS concentration used. The actual binding capacity at pH 5.0 probably lies between 10 and 20 moles TNS per mole α-gliadin.

Turbidity was observed in the α-gliadin chamber in some of the dialysis experiments. This was detectable by the unaided eye at equilibrium binding ratios of about 6 TNS/α-gliadin at pH 3.1, and about 1 TNS/α-gliadin at pH 5.0. The turbidity persisted through the higher binding ratios. Its relation to TNS binding may be through the partial neutralization of the protein's positive charge by the

[3]Estimated from data of Camerman and Jensen, Science 165: 493 (1969).

anionic ligand. The resulting decrease in intermolecular repulsion would then allow aggregation-favoring forces to become dominant. The onset of aggregation causes no abrupt changes in the shape of the binding curve; apparently the apolar area blocked by the aggregation, if any, is relatively small. The probability of such blockage might increase, however, as larger amounts of TNS are bound and induce higher degrees of aggregation.

Equilibrium dialysis experiments with sodium dodecyl sulfate indicate that it is bound more strongly by α-gliadin than is TNS, but that the binding is qualitatively similar to that of TNS in terms of pH dependence and induction of aggregation. These results will be described in a later publication.

DISCUSSION

The experimental work presented here indicates that α-gliadin can bind appreciable amounts of TNS. The increase in fluorescence yield of the TNS that accompanies this binding is interpreted as positive evidence for the existence of exposed apolar regions on the surface of the α-gliadin molecule and probably other gliadins as well. The quantum yield of α-gliadin•TNS fluorescence measured at pH 3.1 suggests a binding environment similar to that presented by bovine serum albumin.

The possible binding forces involved in the interaction of α-gliadin with the organic anion TNS include both electrostatic and hydrophobic components. In related research on the binding of organic anions to bovine serum albumin, Decker and Foster (10) and Ray et al. (7), have concluded that the major portion of the energy of such binding is attributable to apolar interactions. Similar conclusions were reached by Stryer (11), regarding the binding of 1-anilino-8-naphthalenesulfonate ("ANS", a probe structurally similar to TNS) to apomyoglobin and apohemoglobin; and by Daniel and Weber (12), regarding the binding of ANS to bovine serum albumin. In the present study, estimates based on the theory of electrostatic interactions (8, p. 537) suggest that electrostatic contributions determine only a minor portion of the total energy of α-gliadin•TNS binding. It is thus likely that the binding of TNS to α-gliadin is strongly influenced by apolar interactions.

The determinations that purified α-gliadin can bind appreciable amounts of TNS are in general agreement with recent analyses of the binding of natural flour lipids to fractionated gluten components (13,14), which indicate that gliadins mainly bind polar lipids.

The behavior of α-gliadin is pH sensitive in a number of ways in the region pH 3.1 to 5.0. For example, upon increase in the pH from 3.1 to 5.0, the degree of aggregation of α-gliadin increases, whereas the α-gliadin•TNS total fluorescence yield, the binding affinity, and the apparent binding capacity of α-gliadin for TNS decrease. Some commonness of origin of these sensitivities is suggested, for example, by similar approximately tenfold decreases of α-gliadin•TNS binding affinity and total fluorescence yield as pH is changed from 3.1 to 5.0. All the changes noted seem to indicate a decrease in accessibility of the protein's apolar regions with increasing pH. Even at pH 5.0, however, α-gliadin has a significant amount of exposed apolar area.

The pH sensitivity mentioned above may have as possible causes protein

conformational change, protein aggregation, and a change of electrostatic effects. A conformational change could cause changes in both the binding capacity and binding affinity of α-gliadin for the potential ligands. The primary potential of aggregation phenomenon would be for reduction of total binding *capacity* through physical obstruction of previously accessible apolar areas. Strong local electrostatic effects, a possible result of increased carboxyl ionization, would most likely reduce apparent binding *affinities* through increased repulsion of TNS. Positive evidence for a pH mediated conformational change in α-gliadin has been reported by Kasarda et al. (15) on the basis of circular dichroism and optical rotatory dispersion studies. They detected a slight increase in α-gliadin α-helix content and intensification of aromatic dichroism bands upon changing from pH 3.0 to 5.0. These changes, indicative of a "tightening" of the protein's conformation, correlate well with the decrease in α-gliadin•TNS binding that occurs between pH 3.1 and 5.0, and the phenomena probably are directly related. The data from the present study also show a parallel between the changes in binding parameters and the degree of protein aggregation.

The results of this study provide no firm basis for differentiating between effects of aggregation and of conformational change; however, the occurrence of a greater change of binding *affinity* (∿10X) with pH than of apparent binding capacity (<1.5X) with pH suggests that protein conformational change is the major cause of the change in binding. More precise definition of these effects may be aided by study of the role of electrostatic factors in the protein-ligand interaction.

It has been demonstrated by Swanson and Andrews (16) that surface-active agents such as sodium dodecyl sulfate alter the mixing characteristics of doughs, mainly through interaction with gluten proteins (17). Some currently popular dough conditioners have a "polar head•apolar tail" configuration similar to that of SDS and TNS, and their action probably involves apolar binding to proteins. The protein•TNS fluorescence technique may thus find some application in the study of protein•lipid interactions and their effects on dough rheology.

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