The Modification of Wheat Flour Proteins with Succinic Anhydride

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ABSTRACT

Treatment of wheat flour with succinic anhydride in aqueous dioxane suspension results in the conversion of 95% of the protein to derivatives soluble in water. Decreasing the pH below 5.0 results in precipitation of most of these derivatives. A substantial fraction was precipitated at 1% Na_2SO_4 concentration, but another fraction was insensitive to high salt concentrations. Sensitivity to low pH and salt concentration diminished as the dioxane content of the system increased. G-100 Sephadex chromatography and viscosity studies indicated that the succinylated derivatives are more extensively dissociated in solution than untreated flour proteins in a dilute acetic acid extract. Polyacrylamide gel electrophoresis and ultracentrifugal analysis indicated that some association phenomena still occur unless the derivatives are dissolved in concentrated urea solutions. Gel electrophoresis of native and succinylated solutions of serum albumin, pepsin, and lysozyme indicated that the succinylation of a single protein species is likely to produce a heterogeneous product.

The use of succinic anhydride as a modifying agent in the study of proteins was first described by Habeeb et al. (1). Since then the technique has been investigated and discussed by several others (e.g., 2–5). Succinic anhydride reacts with free amino groups, tyrosyl hydroxyl groups, and sulfhydryl groups in proteins, forming amide, ester, and thioester linkages respectively. The tyrosyl ester and thioester linkages are hydrolyzed spontaneously in aqueous media within a few hours (3,4). Under the conditions employed, little apparent reaction occurs with the alcoholic hydroxyl groups of serine and threonine (1,4).

As a result of the reaction, each amino group with its potential positive charge is masked, while at the same time an additional carboxyl group is introduced. Thus the pattern of inter- and intramolecular electrostatic forces is markedly altered.

Although there have been some recent successes in the isolation of pure protein components from flour (e.g., 6,7), two problems have always hampered progress toward the complete separation and characterization of these proteins. The first is the limited range of conditions under which many of them can be brought into solution; the second is the strong tendency for flour proteins to interact and form complexes with one another. The present study was motivated upon the hypothesis that the succinylated derivatives may be more readily solubilized and separated from one another than the unmodified flour proteins.

The work to be described has shown that a mixture of succinylated flour proteins is soluble under a different range of conditions than the unmodified proteins. The effect of pH and salt concentration on the solubility of these derivatives has been examined. Comparative studies involving viscosity measurements, gel chromatography, gel electrophoresis, and analytical ultracentrifugation have been performed.

MATERIALS AND METHODS

All chemicals were reagent grade unless otherwise specified. Two samples of untreated flour were used. A bread flour was milled from the HRS variety Manitou with ash content of 0.39% and a crude protein (N × 5.7) of 12.4%. A soft wheat flour was milled from the variety Pitic, with ash and crude protein contents of 0.35

and 10.8% respectively. Milling was performed on an Allis-Chalmers experimental mill. Commercially prepared crystalline samples of bovine serum albumin (BSA), pepsin, and hen egg lysozyme were purchased from Pentex, Nutritional Biochemical Corporation, and Calbiochem respectively. The 1,4-dioxane was freshly distilled over NaOH pellets before use; 2-mercaptoethanol and acrylonitrile were also freshly distilled.

Extraction and Separation of Flour Protein Components

For some experiments an extract was prepared by stirring flour and 0.2M acetic acid in a 1:5 ratio w./v. for 30 min. The insoluble residue was removed by centrifugation. Starting with a similar extract, a solution containing mainly the glutenin and gliadin components of flour was prepared by adding solid NaCl to a final concentration of 0.2M, and adjusting to pH 7.0 with 2.0M NaOH. The resulting precipitate was recovered by centrifugation and redissolved in 0.2M acetic acid.

Chemical Modification of Proteins

Solid succinic anhydride was added in successive, small portions to aqueous protein solutions (or 1:5 w./v. flour-water suspensions). The pH was maintained near 8.5 by the addition of either 1.0 or 2.0M NaOH solution, from a buret, over a period of 30 min. (1). The reaction vessel was stirred continuously at ice bath temperature. The total amount of added succinic anhydride was a 10- to 20-fold excess based on the calculated concentration of amino groups in the protein sample. A modification of the procedure was to gradually add from a buret a 5% w./v. solution of succinic anhydride in 1,4-dioxane in place of the solid reagent. With flour suspensions, the insoluble residue was removed by centrifugation. Extensive dialysis of the supernatant vs. water was used to remove dioxane and other low-molecular-weight components. Solutions were concentrated by pervaporation (8). For some experiments the dialyzed solution was lyophilized and further solutions of the desired concentration were made up from the dry material.

Reduction and alkylation of protein disulfide groups were performed in solutions containing 8.0M urea, using 2-mercaptoethanol and acrylonitrile according to the procedure used by Woychik et al. (9).

Analytical Methods

Nitrogen and crude protein concentration were determined by the Kjeldahl method (10) and by the biuret method employing reagent B of Pinckney (11). The latter method was standardized against the former. The ninhydrin procedure of Rosen (12,13) was employed to estimate the extent to which the free amino groups of the proteins in solution had reacted with the succinic anhydride. A Carey Model 14 recording spectrophotometer was used in measuring the UV and visible spectra.

In determining the effect of pH on the solubility of succinylated proteins, 0.05N HCl was gradually added, with stirring, to solutions of these derivatives until precipitation began. The insoluble material was removed by centrifugation and the protein remaining in aliquots of the solution was measured. Continuing this procedure, by successive additions of HCl, the solubility was measured over a range down to pH 3.0. Similarly, the effect of salt concentration was determined by successively adding known amounts of solid Na_2SO_4 to the succinylated protein

solutions. At each stage the mixture was stirred, centrifuged, and aliquots of the supernatant taken for protein analysis. Centrifugation was routinely performed with the high-speed attachment of an International PR2 refrigerated centrifuge for 15 min. at approximately 15,000 × g.

Solution viscosities were measured with an Ostwald Viscosimeter having a flow time of 71 sec. with water at 20°. A series of solutions containing decreasing concentrations of the protein in question was measured, and reduced viscosities were calculated as described by Mysels (14). Protein solutions were in 0.01M phosphate buffer, pH 7.0, except for untreated flour proteins which were in 0.2M acetic acid.

Chromatography on Sephadex G-100 was carried out using a 22 mm. diameter column with a bed height of 105 cm. The column was prepared according to the procedure recommended by the manufacturer[1] and operated at a flow rate of 0.33 ml./min. The hydraulic head required to maintain this flow rate gradually increased from 19 to 27 cm. of water during the course of several experiments. The sample volume ranged from 1 to 5 ml., and 5-ml. fractions were collected. Elution was with 0.01M phosphate buffer, pH 7.0, except for untreated flour proteins which were eluted with 0.2M acetic acid.

Polyacrylamide gel electrophoresis was performed in an apparatus similar to that described by Lawrence et al. (15). The gels (5 to 7% concentration) were prepared according to the procedure of Raymond and Wang (16). Protein bands were stained by immersion in a 0.25% w./v. solution of Coomassie Brilliant Blue R-250[2] (17) in a solvent mixture of methanol, water, and acetic acid (25:75:10), and destained by washing in repeated changes of the solvent mixture. Buffer systems included 0.03M phosphate, pH 7.0, and 0.05M veronal, pH 8.5. Maximum applied potential was 15 v./cm., and most runs were 4 hr. or less.

Sedemintation coefficients were determined with a Spinco model E analytical ultracentrifuge and were not corrected for concentration effects. For molecular-weight determinations, the meniscus depletion method was used (18).

RESULTS AND DISCUSSION

Effect of Succinic Anhydride on the Extent of Flour N Extraction

Treatment of a flour-water suspension with solid succinic anhydride resulted in the solubilization of only 20% of the flour nitrogen. This is quite similar to the degree of nitrogen extraction obtainable with water alone. However, treatment with a 5% w./v. solution of succinic anhydride in 1,4-dioxane, to a final concentration of 30% dioxane, gave much higher results. In repeated trials, 89 to 95% of the N in the bread flour and 92 to 97% in the soft wheat flour was solubilized. These values were calculated on the assumption that the liquid remaining with the wet insoluble residue contained the same concentration of dissolved protein as the extract. In all cases, the residue was quite gelatinous. Approximately one-third of the liquid remained with the residue after centrifugation, even though the final ratio of flour to extractant was 1:10. In one experiment, the residue from the succinylation

[1] Pharmacia Fine Chemicals, Uppsala, Sweden.

[2] The use of Coomassie Brilliant Blue as a protein dye rather than the more conventional Amido Black (16) was adopted because the latter did not stain succinylated proteins at all well. As Amido Black is an anionic dye, its failure to work well under these conditions is not surprising.

procedure was further extracted with water. A recovery of 89% in the original extraction was increased to 97% by the second extraction, and it was clearly demonstrated that the nitrogen remaining in the wet residue could be brought into aqueous solution. The efficiency of nitrogen extraction depended upon the purity of the dioxane. When a technical grade was substituted for the more highly purified solvent, the extent of solubilization was lower, ranging from 80 to 85%.

Aqueous dioxane, in the absence of any succinic anhydride, was a much better extractant for flour proteins than water. The effect of dioxane concentration on the extent of protein extraction is shown in Fig. 1. With 30% dioxane, 60% of the flour N can be brought into solution. It appears that the solubility of flour proteins in aqueous dioxane is similar to that observed in aqueous alcohol. The dioxane can facilitate the succinylation reaction by exposing the reactive sites to attack by the reagent in a flour suspension.

Extent of Reaction of Succinic Anhydride with Flour Proteins

The ratio of the ninhydrin color to the total N content was determined for solutions of succinylated and untreated flour proteins. For an extract obtained by the treatment of an aqueous flour suspension with a 5% w./v. solution of succinic anhydride in 1,4-dioxane, this ratio was only one-tenth that observed for a 30% aqueous dioxane extract of the same flour. Furthermore, the visible spectrum of the products of the ninhydrin reaction with succinylated flour proteins was considerably different from that observed for the untreated extract. The color of the latter resembled, to a much greater extent, the typical ninhydrin color of the free amino acids. Consequently, it has been estimated that the extent of reaction of flour amino groups with succinic anhydride was over 90% and might well be complete.

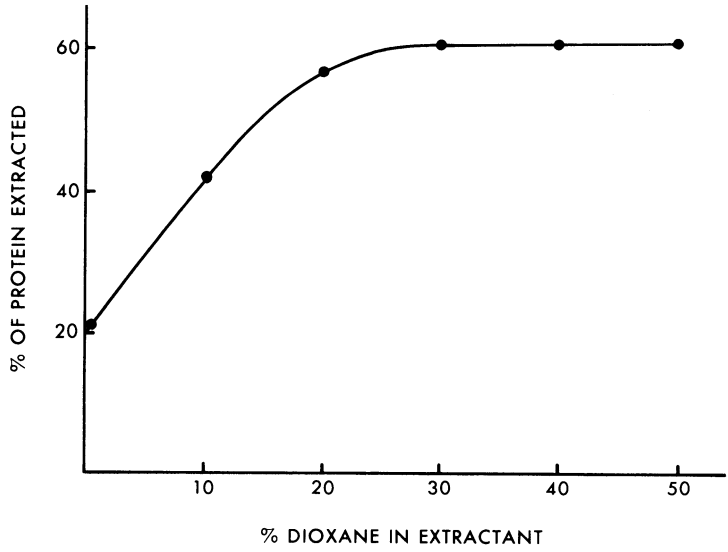

Fig. 1. Effect of dioxane concentration on the extent to which flour proteins may be solubilized in aqueous dioxane. The ratio of flour to extractant was 1:5 w./v.

After flour proteins were treated with succinic anhydride, the ultraviolet spectra of the resulting derivatives underwent a gradual change over a period of approximately 4 hr. This spectral change was in qualitative agreement with that which would be expected as a result of the spontaneous hydrolysis of the tyrosyl succinate linkages (3,4).

Factors Affecting the Solubility of Succinylated Flour Protein

Removal of dioxane and excess reagents by exhaustive dialysis against water did not cause any precipitation of the succinylated flour protein. Only 4% of the N in the extract was removed by dialysis. It was also observed that 5 min. of heating in a boiling-water bath caused no coagulation of the succinylated protein in aqueous solution.

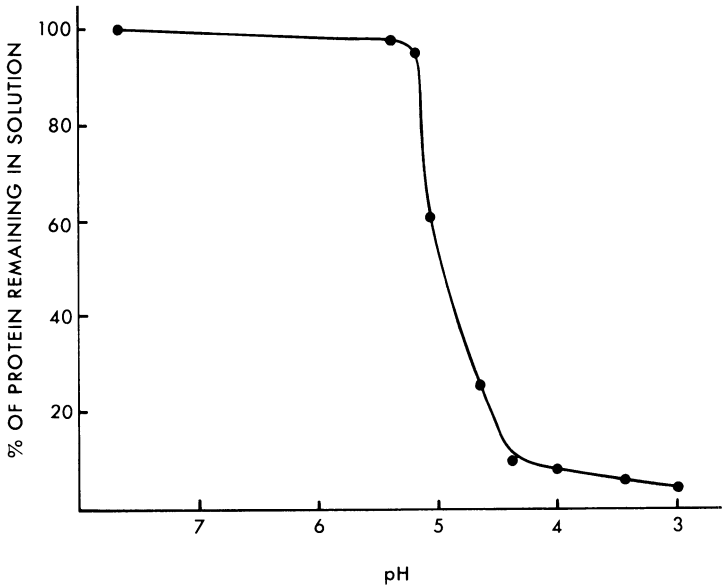

Fig. 2. Effect of pH on the solubility of succinylated bread flour proteins. The initial protein concentration was approximately 0.5%. Adjustment of pH with dilute HCl caused a 30% increase in volume of solution, by pH 4.5.

The data in Fig. 2 illustrate that pH had a pronounced effect on the solubility of these derivatives in water. Precipitation began as the pH was lowered to approximately 5.0 and 90% of the material soluble under neutral conditions was precipitated at pH 4.5. Thus it appears that water solubility is dependent upon a high degree of ionization of the carboxyl groups. This sensitivity to pH was greatly diminished in solutions that contained dioxane. With 30% dioxane, only 40% of the succinylated protein was precipitated at pH 3.0.

The effects of increasing salt concentration on the solubility of the succinylated flour protein derivatives under four different sets of conditions are shown in Fig. 3. In a 0.1M phosphate buffer, pH 7.0, a substantial fraction of the material was

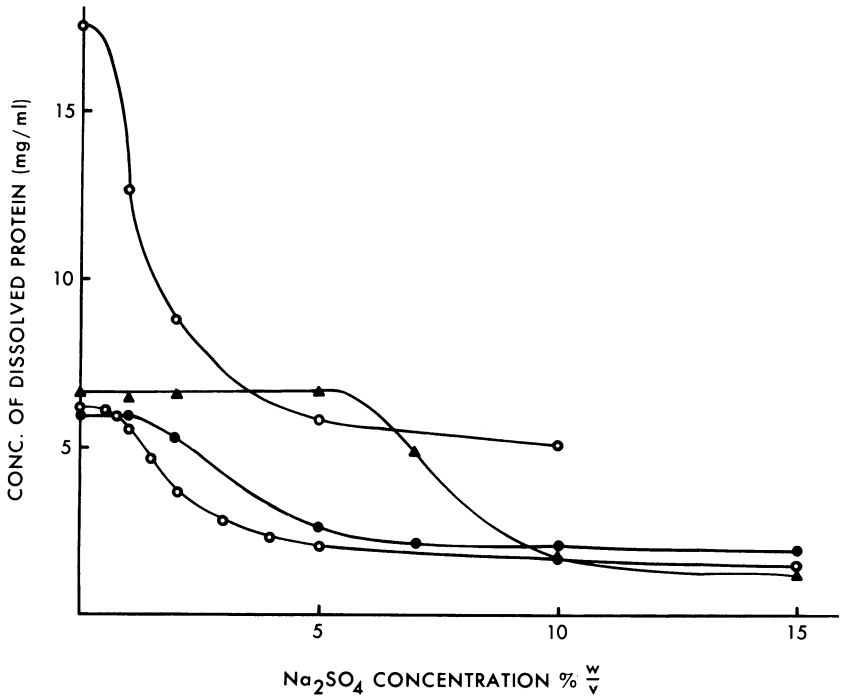

Fig. 3. Effect of salt concentration on the solubility of succinylated bread flour proteins. ○: pH 7.0 in 0.1M phosphate buffer at two levels of initial protein concentration. ●: pH 9.0 in 0.1M borate buffer. ▲: pH 7.0 in 0.1M phosphate buffer containing 20% v./v. dioxane.

precipitated by relatively low concentrations of Na_2SO_4. Another fraction remained soluble at salt concentrations that approached saturation. The salt-sensitive fraction was further influenced by changing the pH value and particularly by the presence of dioxane. Higher salt concentrations were required at pH 9.0 than at pH 7.0 to cause the same extent of precipitation. In the presence of 20% dioxane, much higher salt concentrations were required to cause any precipitation. The differing effects of ionic strength on the solubility of the various protein components in untreated flour are well known. It appears that the factors responsible for these differences are retained, to some degree, in the succinylated derivatives.

Sephadex Chromatography

To obtain information on how the succinylation of flour proteins affects their tendency to associate and form complexes with one another, the behavior of solutions on a Sephadex G-100 column was examined. Some preliminary experiments with BSA and pepsin, both succinylated and untreated, were performed to test the column and to determine the effects of succinylation on chromatographic behavior. The results are shown in Fig. 4 (A and B). The untreated BSA gave two peaks, the minor one probably being the dimer, which commonly occurs in BSA preparations (19). With untreated pepsin a single peak appears,

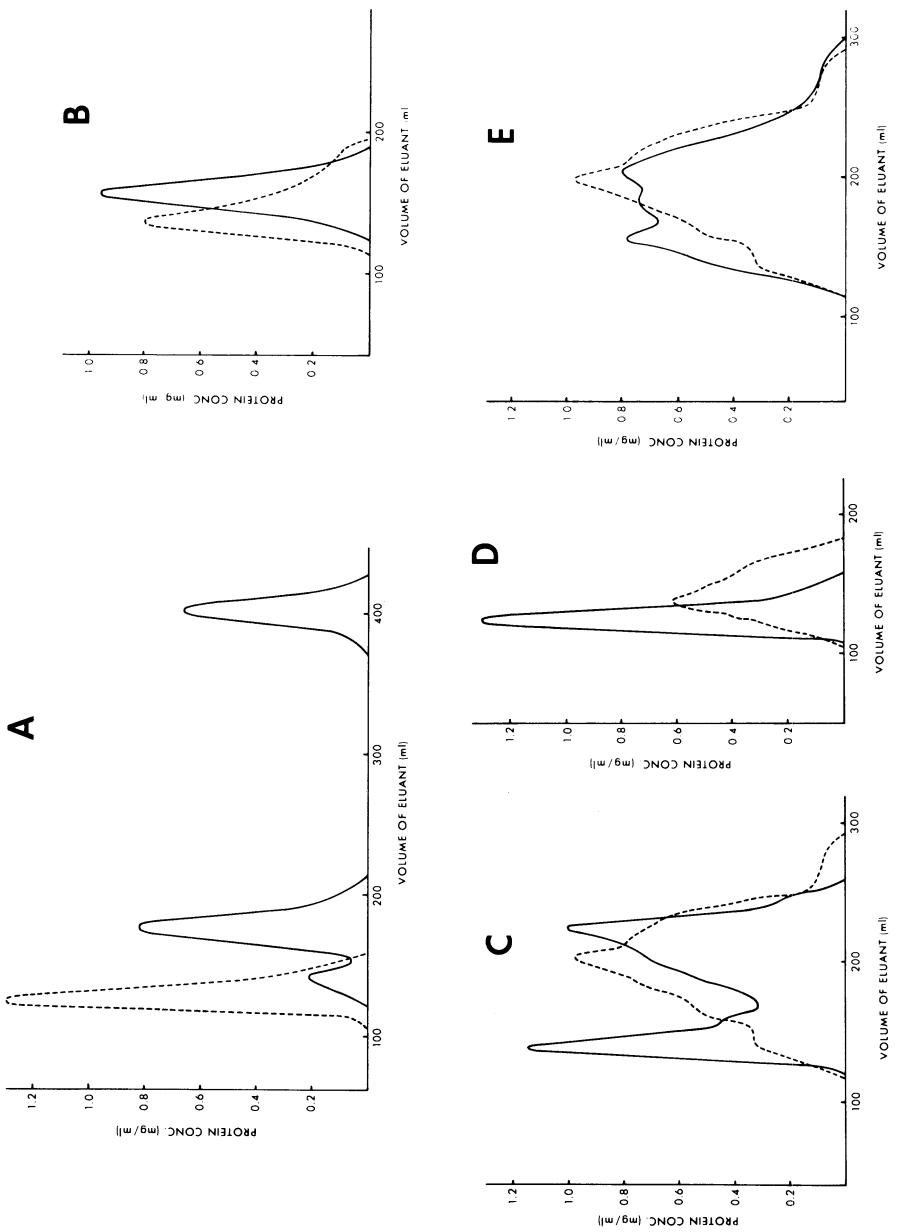

Fig. 4. Chromatography on Sephadex G-100.————, solutions of untreated proteins; ------, the same protein after succinylation, except in E. The peak at 400 ml. in A is that for glycylglycine, which was routinely added as a marker for use in column calibration. A, bovine serum albumin; B, pepsin; C, a 0.2M acetic acid extract of bread flour; D, a high-molecular-weight fraction isolated from the acetic acid extract by prior chromatography on the same column; E, a comparison of the succinylated acetic acid extract (------) with the succinylated derivatives solubilized by treatment of a suspension of the same flour with succinic anhydride in dioxane solution (————).

although it emerges considerably earlier than expected on the basis of its molecular weight. This behavior has been noted and rationalized by Chao and Einstein (20). In both cases the succinylated derivatives emerge as single peaks much earlier than the untreated proteins, almost coinciding with the void volume of the column. It would appear that the increase in negative charge on these derivatives, and the resulting increase in intramolecular, electrostatic repulsive forces, have caused the molecules to unfold and expand to larger volumes.

Chromatograms that show comparisons of succinylated and untreated wheat flour protein solutions are presented in Fig. 4 (C,D, and E). The late-emerging, relatively low-molecular-weight components that appeared in the acetic acid extract tend to be eluted somewhat earlier after succinylation (Fig. 4C), as was the case with the reference proteins. However, the proportion of material that emerged in the void volume after the extract had been succinylated was much smaller, and much of the apparently high-molecular-weight protein had been modified by the treatment so that it was eluted somewhat later. This is interpreted to indicate that succinylation promotes some degree of dissociation for those protein complexes which exist in untreated acetic acid extracts. This conclusion is supported by the comparison shown in Fig. 4D between an untreated, apparently high-molecular-weight fraction isolated from an acetic acid extract and the same fraction after succinylation.

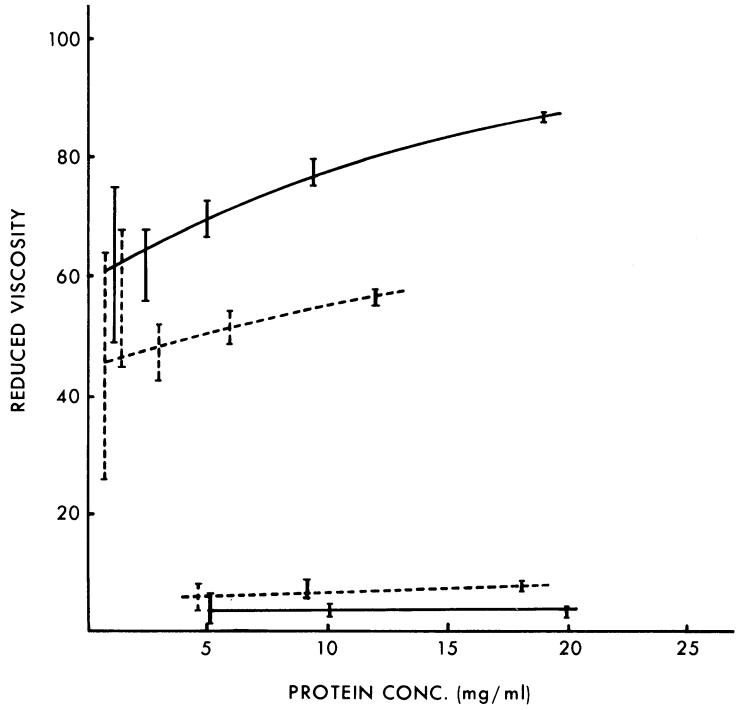

Fig. 5. Reduced viscosity of BSA (————) and pepsin (------) before and after succinylation. The upper two curves are for the succinylated derivatives. Temperature was 20°C.

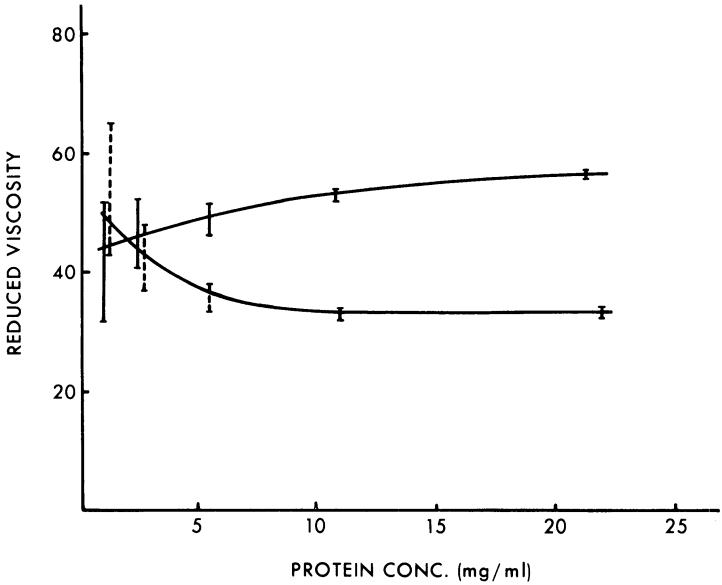

Fig. 6. Reduced viscosity of a preparation consisting mainly of gliadin and glutenin from bread flour before succinylation (lower line) and after succinylation. The temperature was 20°C.

Succinylation of a flour suspension resulted in a higher proportion of the early emerging species in comparison to that observed in the succinylated acetic acid extract (Fig. 4E).

Viscosity Studies

The reduced viscosity of BSA and pepsin solutions was much lower in the native state than it was after succinylation, as shown in Fig. 5. An untreated preparation consisting mainly of gliadin and glutenin components of wheat flour[3] in 0.2M acetic acid solution had quite a high reduced viscosity, and succinylation resulted in a relatively small increase to approximately the same level as succinylated pepsin (Fig. 6).

These results seem consistent with the conclusions reached from the results of Sephadex chromatography. The relatively small increase in flour protein viscosity resulting from the reaction with excess succinic anhydride can readily be explained. The tendency for the viscosity to increase as the polypeptide chains unfold is counteracted, to a large extent, by the dissociation of the protein complexes that exist in the untreated extract.

Gel Electrophoresis

Preliminary electrophoresis experiments on polyacrylamide gels, with pepsin and lysozyme, showed only one protein band at pH 7.0, whereas the BSA preparation showed two minor, slower-moving bands in addition to the main band.

[3]This preparation was used because it contained a relatively small amount of nonprotein material. Interference due to the effects of such nonprotein material on solution viscosity was thereby minimized.

After treatment with excess succinic anhydride each of these proteins showed several strong bands. With BSA, treatment with a small amount of succinic anhydride produced a rapidly migrating band that disappeared with further treatment. It would appear that when BSA is treated with small amounts of the reagent, a derivative with enhanced negative charge is produced to account for the high mobility. But the unfolding of the protein that results from further succinylation eventually slows the migration rate, because of the sieving effect of the gel. The use of a hundredfold rather than a tenfold excess of reagent caused no further change in the electrophoretic band patterns.

With lysozyme, which behaves as a cation at pH 7, reaction with succinic anhydride in aqueous dioxane solution produced only positively charged derivatives. Reaction was more complete and only anionic derivatives were produced when aqueous lysozyme solutions were treated with solid succinic anhydride. With BSA and pepsin the method of treatment had negligible effects on the band patterns obtained.

Reduction and alkylation of the disulfide bonds in succinylated lysozyme, followed by further treatment with solid succinic anhydride, did result in a homogeneous product as evidenced by a single band in the gel electrophoresis pattern. However, the same treatment of either pepsin or BSA resulted in highly heterogeneous products by the same criteria. Thus it appears that under the conditions employed, it is improbable that the succinylation of a single protein component will produce a single derivative. The unfortunate consequence is that the potential value of this procedure for studying flour proteins is much less than it would otherwise be.

Electrophoresis of succinylated flour proteins at pH 7.0 produced a pattern of about four very faint bands against high levels of background staining and streaking, plus a substantial amount of material of such high apparent molecular volume that it was unable to migrate through the gel. Instead, it produced a zone of intense staining at the edge of the slot where the sample was inserted. Only slightly better results were achieved in 0.1M veronal buffer at pH 8.5. When succinylated flour proteins were reduced, alkylated and further treated with solid succinic anhydride, and subjected to gel electrophoresis in the pH 8.5 veronal buffer in 3M urea, a clear pattern of 17 bands was obtained. A sketch of the pattern is shown in Fig. 7. Omission of urea resulted in a recurrence of the streaky patterns and staining at the edges of the slot where the sample was inserted. Thus it appears that in spite of the evidence obtained from Sephadex chromatography, the succinylated flour proteins retain a considerable tendency to associate and form complexes, if dissociating reagents such as urea are omitted from the system.

The method of gel electrophoresis is capable of resolving a large number of components. Because of the strong indication that individual proteins may each produce several succinylated derivatives, it is rather surprising that the pattern shown in Fig. 7 does not show many more components. As a comparison, more than 30 bands can be resolved upon gel electrophoresis of wheat flour proteins in aluminum lactate buffer systems (21).

Ultracentrifugal Analysis

The molecular-weight distribution of reduced, alkylated,and succinylated wheat flour proteins was furthur investigated by sedimentation velocity analysis. In the absence of urea, a major component of S_{20w} 2.07 S and a minor component

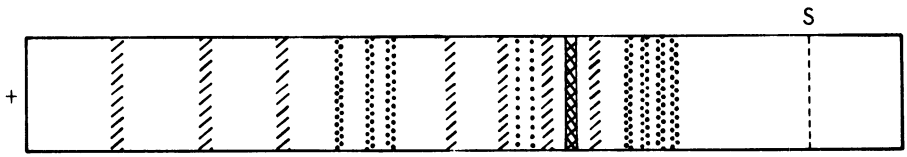

Fig. 7. Pattern obtained on polyacrylamide gel electrophoresis of reduced, alkylated, and succinylated bread flour proteins in veronal buffer containing 3.0M urea, pH 8.5. S marks the position where the sample was inserted.

representing approximately 20% of the total with an S_{20w} value of 7.68 S were observed. In the presence of 3.5M urea there appeared only one component with an S_{20w} of 1.24 S. The molecular weight of the major component in the absence of urea was calculated to be 16,600, while in 3.5M urea a value of 11,000 was determined (18). These results indicate that urea does promote the dissociation of complex species which exist in its absence. The molecular-weight values seem low, in comparison to those reported by others (22,23) but agreement with the results of Stanley and co-workers (24), using phenol-acetic acid-water as a dissociating solvent, is fairly good.

Acknowledgments

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