white was appreciably similar to that of fresh thick white homogenized with barbital buffer (shown in Figure 2).

Figure 4 shows the elution pattern on Sepharose 4B column of fresh thick white reduced with 0.01 M mercaptoethanol. The protein profile of ovomucin fraction shows another broader peak of fraction no. 30–42, due to the disulfide cleavage of α-ovomucin, although this broader peak did not emerge in the elution patterns of stored egg white. On the other hand, the sialic acid rich ovomucin, reduced β-ovmucin, was still present in the void volume fraction, suggesting that it was of high molecular weight, although the peak slightly shifted to the lower molecular side.

DISCUSSION

Our hypothesis on a mechanism of thinning was confirmed by following the behavior of aggregated and disaggregated ovomucin during storage of egg white. The carbohydrate-rich ovomucin (β-ovomucin) disaggregatd from ovomucin gel and the carbohydrate-poor ovomucin (α-ovomucin) remained insoluble during egg white thinning. It is an important finding that α-ovomucin polymerized by disulfide bonds remained insoluble for a long-time storage. This is apparently contrary to the hypothesis that ovomucin is depolymerized by the reduction or alkaline hydrolysis of disulfide bonds during thinning.

On the other hand, the ovomucin elution pattern on Sepharose 4B of thinned egg white obtained after 20-day storage was similar to that of fresh thick white homogenized with barbital buffer, pH 8.6, rather than that of fresh thick white in the presence of a reducing reagent.

These results suggest that noncovalent disaggregation of ovomucin occurs during natural thinning, without disulfide cleavage of ovomucin. The ovomucin gel structure of the thick white may be held by noncovalent bonds that are unstable to mild homogenization in mild alkaline solution rather than by the disulfide bond in aggregated α-ovomucin that is not disaggregated during thinning.

Further studies are now being carried out to determine the molecular weight of disaggregated ovomucin during thinning.

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Hemoglobin as a Binding Substrate in the Quantitative Analysis of Plant Tannins

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The use of hemoglobin derived from fresh bovine blood as a binding substrate in protein precipitation of tannins is described. The use of a chromoprotein obviates the need for visualizing either precipitated protein or substances remaining in the supernatant. When the appropriate initial hemoglobin concentration is chosen, the relationship between absorbance of hemoglobin remaining in the supernatant and tannin concentration is linear over a wide range of tannin concentrations and for several tannin types. The binding process is relatively unaffected by solution pH. Methods combining the precipitation of hemoglobin with other techniques, such as the Folin–Denis assay, may help elucidate the details of the binding process.

Tannins are polyphenolic compounds which form stable complexes with proteins (Swain, 1979). They are nearly ubiquitous in woody plants (Bate-Smith and Metcalf, 1957) and are widespread in various tissues of herbaceous plants as well, including economically important species (Swain and Hillis, 1959; Chan et al., 1978; Hagerman and Cutler, 1978). The proposed biological actions of tannins include complexing with plant proteins, reducing the digestibility of tissues to insect or vertebrate herbivores, complexing with digestive enzymes (Feeny, 1968, 1969; Williams, 1963; Goldstein and Swain, 1965), or acting as behavioral antifeedants (Bate-Smith, 1973; Goldstein and Swain, 1963). Thus, several techniques which estimate tannin content of plant material by determining the affinity of plant extracts for proteins have appeared in the literature (Bate-Smith, 1973; Chan et al., 1978; Hagerman and Butler, 1978; Swain, 1979; Walter and Purcell, 1979; Boudini et al., 1980).

A useful tannin assay should have the characteristics of simplicity and adaptability to small-sized samples and should not require specialized equipment (Bate-Smith, 1973). The use of the chromoprotein hemoglobin as a binding substrate (Bate-Smith, 1973) meets these requirements but has only been employed for qualitative taxonomic work [e.g., Bate-Smith (1977)]. Hemoglobin concentration can be measured directly by visual spectrophotometry and can be used in the analysis of single leaves (Bate-Smith, 1973; Swain, 1979). In this communication we report on the use of hemoglobin in a quantitative assay for plant tannins. We report here on the

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usefulness of the technique in the quantitative assay of several tannin types and the influence of pH on tannin–protein binding.

MATERIALS AND METHODS

Materials. Reagent-grade chemicals (Fisher) were used throughout.

Hemoglobin solutions were prepared by collecting steer blood fresh at slaughter. Cells were lysed immediately by addition of cold (5 °C) distilled water. The mixture was returned to the laboratory on ice, where it was centrifuged to produce a cell membrane free solution which remains stable for 90 h if stored at 5 °C. Tannic acid (Fisher), wattle condensed tannin, and sumac and chestnut hydrolyzable tannin (Leon Monnier, Inc.) standards were made up in distilled water. New standards were prepared for each batch of blood and were used within 24 h.

Crude tannin extracts were prepared from mature leaves of yellow birch (*Betula allegheniensis* Britt.) and sugar maple (*Acer saccharum* Marsh) trees growing near Hanover, NH. Leaves were returned to the laboratory on ice, weighed, and quick frozen in liquid nitrogen. They were then ground by hand in 50-mL Ehrlenmyer flasks by using a glass pestle. Methanol–water (1:1 v/v; 35 mL/g of leaf) was added to the flask, and the flasks were placed in a water bath at 77–80 °C for 1 h. In separate experiments, the extraction of the hemoglobin-complexing leaf constituents was found to be essentially complete after 30 min and was independent of the initial leaf particle size. After extraction, all samples were vacuum filtered through No. 1 Whatman paper. The filtrate volume was measured, and the extract was stored for up to 2 weeks after refrigeration in acid-washed bottles until analysis.

Analytical Procedures. Hemoglobin precipitation (Heme test) was measured by using a test solution of 0.5–1.0 mL of tannin solution, 1.0–1.5 mL of distilled water, and 1 mL of hemoglobin solution. All solutions were kept refrigerated until use. The tannin solution was added to the hemoglobin solution in a centrifuge tube by using a vortex mixer to avoid the formation of temporary protein gradients during mixing (Bate-Smith, 1973). After being mixed for 15 s, the test solution was centrifuged at 7500*g* for 30 min. The supernatant was decanted and its absorbance determined at 578 nm on a Perkin-Elmer 257 spectrophotometer. Occasionally, a cloudy supernatant was obtained in leaf extracts. Heating the supernatant to 20 °C in a water bath for 45 s clarified the solution.

Dissociation of tannin–hemoglobin complexes at various pH values was determined by vortexing tannin–hemoglobin pellets of a standard size in aqueous solutions of varying pH prepared with HCl and $NaHCO_3$. The suspension was recentrifuged (4600 rpm for 30 min), and the pipetted supernatant was read at 578 nm to give a measure of dissociation.

The total phenolic contents of solutions were estimated by using the Folin–Denis (FD) procedure described by Swain and Hillis (1959). One milliliter of solution was added to 1 mL of FD reagent; after 3 min, 1 mL of 2 N $NaHCO_3$ was added as a fixative. Absorbance was read after 60 min at 725 nm against a blank of equal parts distilled water, FD reagent, and $NaHCO_3$. Solutions were diluted (typically 1:10 or 1:100) with distilled water to concentrations which gave FD test absorbances of less than 2.0.

RESULTS AND DISCUSSION

The relationship between hemoglobin absorbance and tannin concentration was found to be linear over a large range of tannic acid and wattle tannin concentrations, as well as for a dilution series of sugar maple leaf extract (r values = 0.97, 0.99, and 0.93, respectively). The range of tannin concentrations over which linearity was obtained was found to depend upon the concentration of hemoglobin used in the test solution (Figure 1). At low initial hemoglobin concentrations, precipitation proceeds until the hemoglobin in the test solution is exhausted. At higher initial hemoglobin concentrations, there appears to be threshold concentration of tannic acid necessary for hemoglobin removal, and the concentration at which precipitation is initiated increases with increasing initial hemoglobin concentrations (Figure 1). Although the range of concentrations over which the relationship is linear is greater at high initial hemoglobin concentrations, resolution for low tannin concentrations may be lost. Similar results were obtained for wattle condensed tannins.

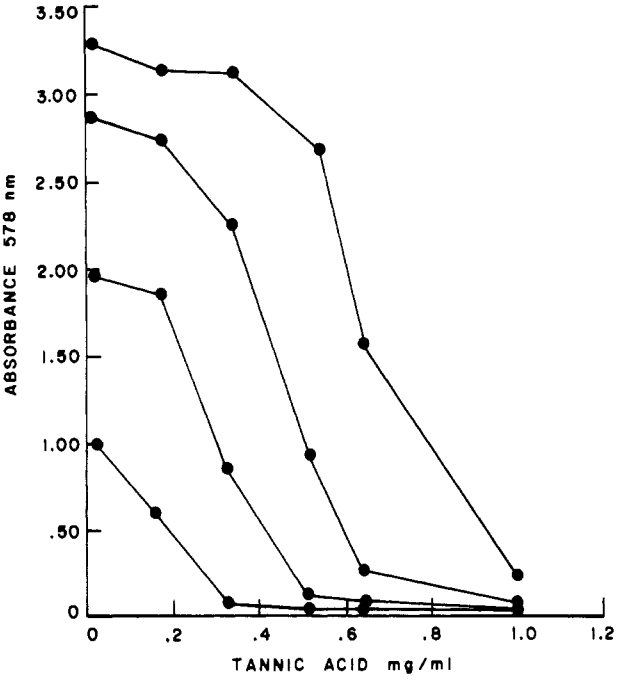

Figure 1. Absorbance of the supernatant at 578 nm after precipitation with tannic acid. Four initial hemoglobin concentrations are shown.

As a consequence, the initial hemoglobin concentration can be adjusted for optimal sensitivity in the range of tannin contents which are to be assayed. In the tree leaf extracts described above, a hemoglobin solution giving a blank absorbance of 2.0 was found useful. Because the dry weight concentration of tannins in such extracts may not be known, a convenient measure of tanning activity is the percent dry weight tannic acid equivalent (TAE), which is defined as the ratio of the sample concentration predicted from the standard curve to the actual dry weight concentration of the leaf material extracted (Bate-Smith, 1973).

The pH of tannin solutions has been shown to influence the degree of protein binding (Goldstein and Swain, 1965; Loomis and Battaile, 1966; Hagerman and Butler, 1978). Hence, differences in protein binding may reflect differences in test conditions rather than differences in tannin concentration. This has led other investigators [i.e., Goldstein and Swain (1965), Hagerman and Butler (1978), and Walter and Purcell (1979)] to buffer test solutions when measuring protein binding. However, protein binding may be influenced by the type of buffer used (Goldstein and Swain, 1965). Our results indicate that several types of hemoglobin–tannin complexes are stable over a wide range of pH values (Figure 2). This stability is attributable to the inherent buffering capacity of the

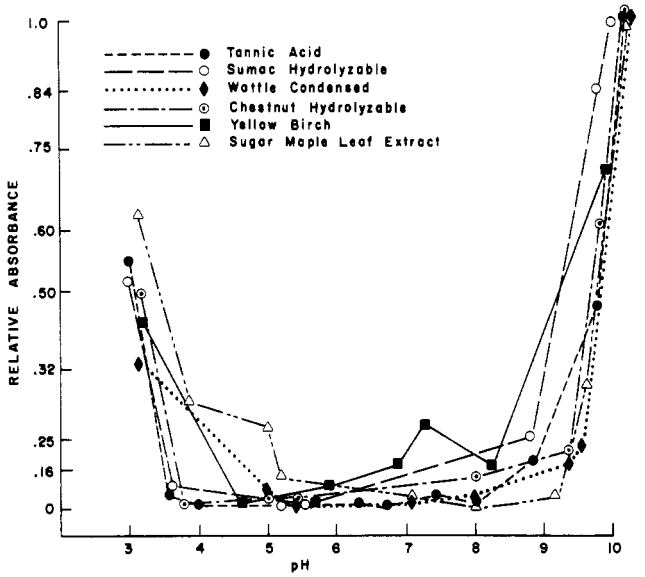

Figure 2. Relative absorbance at 578 nm of the supernatant after dissociating tannin–hemoglobin pellets at various pH values and recentrifuging. Different initial hemoglobin concentrations were used for different tannin types (see the text), so absorbances have been standardized as proportions of their ranges [(absorbance − minimum)/(maximum − minimum)] so they may all be compared to a 0 to 1 scale. 0 = no dissociation; 1 = complete dissociation (all hemoglobin left in the supernatant after centrifuging).

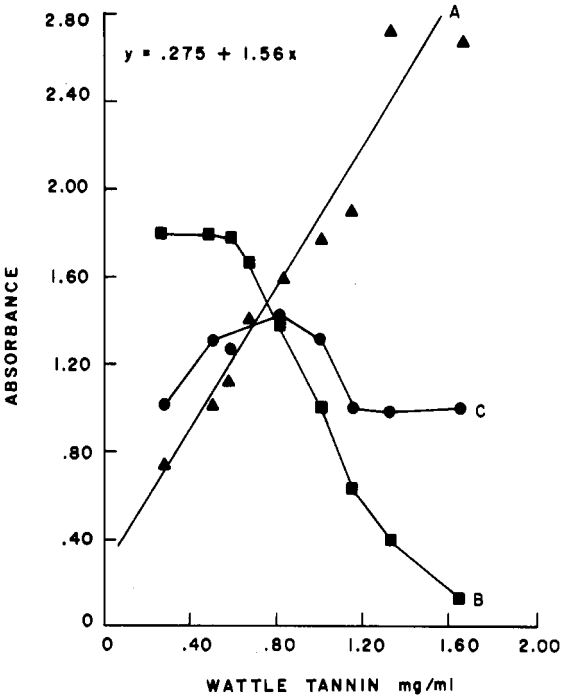

Figure 3. (A) Folin–Denis standard curve plot of absorbance at 725 nm against wattle tannin concentration. (B) Supernatant absorbance at 578 nm after precipitation of hemoglobin by wattle tannin. (C) Supernatant absorbance at 725 nm after addition of Folin–Denis reagent and precipitation of hemoglobin by wattle tannin.

hemoglobin solution: test solutions varying in pH from 4.0 to 8.7 were found to be buffered to pH 6.0–6.2 by the addition of hemoglobin solution. This is also the pH range of maximum binding for all of the tannins examined (Figure 2). All plant extracts and standard solutions were found to lie within this pH range, and thus buffering was not required.

The direct measurement of binding substrate concentrations is one of the principal advantages of the hemoglobin assay. This characteristic not only greatly simplifies quantitative tannin analysis but may also permit an examination of the process of complex formation. One may study this process by measuring both the polyphenol and protein substrate concentrations of the supernatant after tannin–protein precipitates have formed. This is difficult to do when the binding substrate concentration cannot be measured directly, because many reagents used to assay protein concentrations give positive indications with tannins (Robinson, 1979).

Results of a preliminary study utilizing the hemoglobin assay together with Folin–Denis estimates of polyphenols (Figure 3) suggest that tannin–protein binding exhibits cooperativity, in which the first tannin bound greatly facilitates the binding of additional tannins. Comparing the change in hemoglobin concentration with the Folin–Denis activity of the supernatant over a range of wattle condensed tannin concentrations suggest that a tannin threshold is necessary for protein precipitation. The rise in Folin–Denis activity before the precipitation threshold supports this view. In a noncooperative binding situation, one would expect the Folin–Denis estimate of total phenolic concentration to level off after the precipitation threshold has been reached, as further increases in tannins would be removed by precipitation However, the supernatant Folin–Denis activity declines markedly to a low plateau beyond the binding threshold, as binding becomes protein limited. This postthreshold decline indicates that tannin binding is greatly facilitated by the formation of the initial protein–tannin complex. This method of measuring both the amount of protein bound and the residual tannin activity could be extended via different types of tannins and different specific assays of phenolic activity in the supernatant to yield further information about the nature of the tannin–protein interaction.

The use of the chromoprotein, hemoglobin, as a binding substrate for quantitative tannin analyses is both simple and informative. The need for large batches of blood demands proximity to a slaughterhouse (and a willing collector); we found preserved human blood unsuitable because of the addition of anticoagulants. Given a blood source, however, the method is useful over a wide range of tannin types and concentrations, gives reproducible results, sidesteps analytical difficulties arising from the need to visualize other proteins, and can be used with very small tannin quantities or leaf samples. Its solubility in water makes it more convenient than casein, and its apparent buffering capacity reduces variation. It is a suitable alternative to other substrates and offers some unique advantages.

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Protein-Stabilized Emulsions: Effects of Modification on the Emulsifying Activity of Bovine Serum Albumin in a Model System

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A series of laboratory blenders and homogenizers were compared for their capacity to make protein-stabilized emulsions. A Janke-Kunkel blender consistently gave the best emulsions. By use of optimum protein concentrations (0.5%) and temperature (15 °C), the effects of modification on the emulsifying activity (EA) of bovine serum albumin (BSA) over the pH range 2–10 were studied. Reduction of the disulfide bonds reduced the EA, urea (8 M)-eliminated EA, while succinylation significantly enhanced the EA of BSA in the pH range 4–7. These studies indicated the importance of protein structure and charge on emulsifying properties. BSA had superior EA to soy proteins, arachin, β-casein, ovalbumin, and β-lactoglobulin.

Many common foods are broadly classified as emulsions, e.g., milk, ice cream, salad dressing, mayonnaise, and, in some cases, processed meats. In several of these foods proteins serve as the principal emulsifying agents in stabilizing the dispersed oil droplets (Kitchener and Musselwhite, 1968). The criteria frequently used to describe emulsifying properties are emulsifying activity (EA), emulsion capacity (EC), and emulsion stability (ES). The ability of protein to aid in emulsion formation and stabilization is EA, the volume of oil emulsified per unit weight of protein is defined as EC, and the ability of the discrete emulsion droplets to remain dispersed without creaming, flocculating, coalescing, or oiling off is defined as ES. A fixed oil phase volume is necessary for EA and ES, while EC is a function of the amount of oil emulsified.

There is a lot of research concerned with the determination of the emulsifying properties of food proteins. A wide diversity of model systems and varying conditions have been used to determine emulsification properties of numerous proteins, and useful comparisons of methods and results are thus made difficult (Tornberg and Hermansson, 1977; Pearce and Kinsella, 1978; Crenwelge et al., 1974; Acton and Saffle, 1970).

Emulsifying activity reflects the ability of the protein to aid emulsion formation and stabilization of the newly created emulsion (Kitchener and Musselwhite, 1968; Ivey et al., 1970). EA is measured by determining the particle size distribution of the dispersed phase by microscopy, Coulter counting, or spectroturbidity (Walstra et al., 1969). In each procedure an average diameter of the dispersed phase is determined and from these data the interfacial area can be calculated. Results from microscopic techniques take much time and show poor reproducibility, while the Coulter counter method is more reproducible and the lower limit of emulsion particles detected is ~0.7 μm (Walstra et al., 1969).

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The spectroturbidity method is simple, rapid, and theoretically sound (Kerker, 1969) and provides information about the average diameter and particle size distribution. The method is applicable to emulsions with average particle size diameters between 0.2 and 8 μm (Mulder and Walstra, 1974; Walstra et al., 1969). The optical density of diluted emulsions is directly related to the interfacial area (i.e., the surface area of all the droplets) for coarse emulsions (Pearce and Kinsella, 1978).

Several types of blenders and homogenizers and many sizes and shapes of containers have been used in emulsion preparation (Tornberg and Hermansson, 1977; Pearce and Kinsella, 1978; Johnson et al., 1977; Tsai et al., 1970). The valve homogenizer has been used widely (Tornberg and Hermansson, 1977; Berger, 1976; Titus et al., 1968; Klotzek and Leeder, 1966; Sherman, 1965) whereas few emulsion studies have been conducted with sonicators (Tornberg and Hermansson, 1977; Smith and Dairiki, 1975; Higgins and Skanen, 1972). These instruments vary in their ability to form an emusion; i.e., the particle size distribution of the oil droplets vary and frequently factors affecting emulsifying activity are overridden by the characteristics of the equipment used.

For determination of the relative emulsifying properties of proteins and for studying the relationship(s) between protein structure and emulsifying properties, a standardized model system for optimum emulsion formation is needed. Earlier we evaluated a spectroturbidimetric technique for determining emulsifying capacity of proteins (Pearce and Kinsella, 1978). In the present study using bovine serum albumin we compared the relative emulsion-forming characteristics of five laboratory homogenizers and studied the relationship between the structure of BSA and its emulsifying activity.

MATERIALS AND METHODS

Bovine serum albumin (Cohn Fraction V), ovalbumin, β-casein, and β-lactoglobulin were purchased from Sigma Chemical Co. (St. Louis, MO) and used as such. Soya protein isolate was obtained from Ralston Purina Company

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