Re-evaluation of Turbidimetry of Proteins by Use of Aromatic Sulfonic Acids and Chloroacetic Acids

Satoshi Ebina and Yasushi Nagai

From studies on 11 different proteins (including native albumin and albumin with reduced disulfide-bridges) treated with sulfosalicylic, 2-naphthalenesulfonic, toluenesulfonic, dichloroacetic, or trichloroacetic acids, we elucidate the interactions determining the resulting turbidities and other factors affecting turbidities, and we discuss the clinical utility of such turbidimetry. At least three interactions are important in determining turbidity: reduction of positive charges on the protein, hydrogen bonding of the non-ionized chloroacetic acids with the protein, and hydrophobic interaction of the aromatic sulfonic acids with albumin. Turbidity varies appreciably with the species of acid and protein, concentrations of acid, temperature, and standing time after acid is added. We conclude that this technique should be restricted to confirming proteinuria.

Turbidimetry with sulfosalicylic acid (SSA) is still one of the most common means of confirming proteinuria or estimating proteins in cerebrospinal fluid. However, this method is not highly quantitative; the turbidity is significantly affected by the ratio of albumin to globulin, albumin yielding greater turbidity than globulin (1-3). For confirming proteinuria, SSA has been exclusively used rather than trichloroacetic acid (TCA), because TCA gives less turbidity. Nevertheless, TCA may be a more sensitive reagent for the tubular proteinuria than SSA (4, 5) because in this situation the urinary proteins consist mostly of those with low molecular mass (6, 7). It is necessary to re-evaluate this technique, because, as we say, turbidimetry with SSA or TCA is still much used, owing to the high sensitivity of albumin to SSA and the simplicity and speed of this technique, although there are now more precise methods for determining proteins in low concentrations.

To date, numerous attempts have been made to elucidate the ability of these reagents or other precipitants used in turbidimetric analysis to produce equal turbidity with albumin or globulins (1-3, 8-10). These attempts, however, have not ascertained the reason why albumin is far more sensitive to SSA than to TCA or the mechanisms responsible for the sensitivity of various proteins, including albumin, to SSA and TCA.

From this point of view, we have examined the question by using 10 proteins with different relative molecular masses ($M_r$) and isoelectric points (pI)—including albumin and albumin with reduced disulfide-bridges—treated with SSA, TCA, and other organic acids—2-naphthalenesulfonic acid (NSA), toluenesulfonic acid (TSA), and dichloroacetic acid (DCA)—as precipitants. We studied variations in turbidity with temperature, concentration of acid, the turbidities as related to the $M_r$ and pI of the proteins, and the peculiar nature of protein precipitate. On the basis of our findings we discuss the interactions between the proteins and the acids that determine the turbidities.

Materials and Methods

Apparatus. We used a Hitachi spectrophotometer 101 for turbidimetry (with 1-cm light-path cuvets) and an Olympus light-microscope, Model FH, to observe protein precipitates.

Proteins. Proteins used in the study included lysozyme (from egg white; Seikagaku-Kogyo, Co., Tokyo; $M_r = 14,000$, $pI = 11.1$); alpha-lactalbumin (Miles Laboratories, Inc., Kankakee, IL, 16,000, 4.7); beta-lactoglobulin (Miles, 18,000, 5.3); alpha-chymotrypsinogen A (Sigma Chemical Co., St. Louis, MO, 23,000, 9.4); pepsin (Miles, 35,000, 1.0); ovalbumin (Miles, 45,000, 4.9); albumin (bovine serum, Miles, 68,000, 4.9); gamma-globulin (bovine, Miles, 150,000, 5.8-7.3); and fibrinogen (bovine, Miles, 340,000, 5.3). Apo-myoglobin was prepared from myoglobin (from horse heart, Miles, 18,000, 7.3) by the acetone—HCl method (11).

Albumin with reduced disulfide bridges was prepared by reduction with dithiothreitol (12). Albumin was dissolved in 50 mmol/L phosphate buffer, pH 7.4, containing 5 mmol of dithiothreitol per liter, and the solution allowed to stand for 1 h at 20 °C. The resulting albumin with reduced disulfide bridges was subjected to turbidimetry without further modification.

Working solutions of the proteins, except for alpha-lactalbumin and apo-myoglobin, were prepared in the phosphate buffer by adding weighed amounts of the protein, dried under reduced pressure over P$_2$O$_5$, to give final concentrations of 0.5 mg of protein per milliliter.

Working solutions for the other two proteins were prepared as follows. About 50 mg of the dry protein was dissolved in 50 mL of the buffer. The solution, however, contained some insoluble material. After the solution was dialyzed against the same buffer, the insoluble material was removed by centrifugation. The protein concentration was determined by the micro-biuret method (13), and was adjusted to 0.5 mg of protein per milliliter with the same buffer.

Chemicals. SSA, TCA, and DCA were reagent-grade commercial materials (Wako Pure Chemical Ind., Ltd., Osaka). NSA (Nakarai Chemicals, LTD., Kyoto) and TSA (Wako) were recrystallized twice from water and dried under reduced conditions.
pressure over phosphorus pentoxide. All the other chemicals were reagent-grade commercial materials, used without further purification.

Turbidity. Turbidimetry was performed by varying the concentration of the acid up to 3.2 mol/L in the presence of a constant concentration of the protein (0.5 mg protein per 5 mL of reaction mixture). To 1 mL of the working solution, 4 mL of the acid solution was added and mixed gently by inclining the test tube. The resulting turbidity was measured at 500 nm.

Determination of the amount of protein precipitate. The same mixture used for the turbidimetry of lysozyme and pepsin was centrifuged at 0 °C for 30 min at 10 000 × g. The protein precipitated by trichloroacetic acid was dissolved in 5 mL of 1 mmol/L NaOH. The protein precipitated by sulfosalicylic acid was washed three times—with ethanol in the case of pepsin, with 1 mol/L TCA solution in the case of lysozyme—and dissolved in 5 mL of 0.1 mol/L NaOH. The protein concentration of the solution was determined by the method of Lowry et al., with phenol reagent with use of each protein as standard (14).

Light-microscopic observation of protein precipitate. The same mixture used for turbidimetry for pepsin and albumin was allowed to stand at 20 °C overnight in a test tube. The precipitate at the bottom of the tube was placed on a glass slide with a pipette and its particulate form was observed.

Results

We first examined the effect on the turbidities of duration of standing after addition of the acids. Turbidities varied appreciably with time, so we determined them after 20 min in the later experiments. Turbidities from beta-lactoglobulin and albumin were determined with various concentrations of SSA and TCA at three temperatures, 0, 20, and 37 °C, and are given as a function of the concentrations of the acids in Figure 1. The turbidities at any concentration of the acids were significantly affected by the temperature, so we kept the temperature constant (20 °C) in later work.

Turbidities from lysozyme, alpha-lactalbumin, apo-myoglobin, alpha-chymotrypsinogen A, pepsin, ovalbumin, gamma-globulin, and fibrinogen were determined (Figure 2), along with those from the above two proteins (Figure 1). The turbidity curve with SSA differed greatly from that with TCA for all of the proteins. The curve with SSA had a single maximum; as the concentration increased, the turbidity increased—reaching a maximum at about 0.8 mol/L for beta-lactoglobulin and pepsin, about 0.1 mol/L for the others—and then decreased to zero. On the other hand, the curve with TCA had two maxima, the first at about 0.1 to 0.2 mol/L, and as the concentration of TCA was increased to more than 0.3 mol/L (corresponding to about 5 g/dL, a concentration commonly used for the turbidimetry), the peak increased again, reaching a second maximum at about 1.0 to 2.0 mol/L, and then decreased to zero. The maximum with SSA is almost equal to the first maximum with TCA for all of the proteins except albumin.

We examined the maxima from the viewpoint of $M_s$ and pI. Alpha-lactalbumin ($M_s = 16000$, pI = 4.7) and beta-lactoglobulin (18 000, 5.3) had equal maxima, but lysozyme (14 000, 11.1) and apo-myoglobin (18 000, 7.3) had maxima nearly threefold those for the other two proteins. Alpha-chymo-
trypsinogen A (23,000, 9.4) had a large maximum, pepsin (35,000, 1.0) a low one. It is clear from the above examples that the sensitivity of all of the proteins, except albumin, to SSA and TCA in concentrations below 0.3 mol/L depends directly on the pI, not on the Mr.

With concentrations of TCA exceeding 0.3 mol/L, the turbidity reached a second maximum, the level of which was similar for all of the proteins. There is, therefore, some difference in the protein precipitate produced by TCA between the lower and the higher concentrations.

The increase in the turbidity was probably due to a quantitative change in the precipitate, i.e., an increase in the amount of the precipitate. To elucidate this possibility, we determined the amount of precipitate for lysozyme and pepsin in the presence of various concentrations of SSA and TCA; the results are given as a function of concentration of SSA and TCA in Figure 3, and are compared with the corresponding turbidity curves from Figure 2.

For both proteins, the precipitate and turbidity curves with TCA showed no simple relationship, whereas those with SSA were in agreement; excess TCA increased the turbidity, regardless of the constancy in the amount of the precipitate. Hence, the increase in the turbidity is due to a qualitative, not quantitative, change in the precipitate.

Photomicrographs of the precipitate of pepsin and albumin are given in Figure 4, which shows that the precipitate of pepsin with any concentration of SSA and with 0.12 mol/L TCA is composed mostly of light particles, which results in lesser turbidities (Figure 2), while the precipitate of albumin with any concentration of SSA is composed mostly of dark particles, resulting in much greater turbidities (Figure 2). For both of the proteins, as the concentration of TCA increased, the dark particles increased, resulting in increased turbidities (Figure 2). Thus turbidity depends directly on the ratio of the dark particles to the total particles produced.

As shown in Figure 2, the turbidities of albumin with SSA and TCA within the lower concentrations differed greatly from each other, although those of the other proteins were almost equal; albumin was much more sensitive to SSA, similar to lysozyme (pI = 11.1), in spite of its low pI (4.9). This finding shows that urinary, cerebrospinal, or other proteins are far more sensitive to SSA than to TCA in the concentrations usually used for turbidimetry (the lower concentrations) when they are in the environment of a large amount of albumin, and are almost equally sensitive to SSA and to TCA when with a small amount of albumin; this confirms the observations of many authors that the sensitivity is significantly affected by the albumin/globulin ratio (1–3), and shows that the suggestion put forward by several authors (4, 5)—that TCA may be a more sensitive reagent for tubular proteinuria than is SSA—resulted largely from the minor presence of albumin rather than the major presence of proteins of low molecular mass in tubular proteinuria. On the contrary, a question remained as to why albumin is far more sensitive to SSA than to TCA in the lower concentrations.

To answer this question, we used some other acids—NSA, TSA, and DCA—with albumin and beta-lactoglobulin and compared the results (Figure 5) with SSA and TCA (Figure 2). NSA and TSA give high turbidity with albumin, as does SSA, and DCA gives low turbidity, as does TCA, with low concentrations. All of the acids give equally low turbidity with beta-lactoglobulin, except for high concentrations of TCA and DCA. Therefore, according to the sensitivity with which albumin is affected, these acids can be divided into two groups: aromatic sulfonic acids, SSA, NSA, and TSA; chloroaacetic acids, TCA, and DCA.

The high sensitivity of albumin to the first group probably is intimately related to its structure. We elucidated this possibility by examining the turbidities produced by SSA and TCA with albumin with reduced disulfide bridges. The turbidity curves for the reduced albumin (prepared according to Materials and Methods) are given in Figure 6, those for al-
bumin in Figure 2. The reduction caused a remarkable change in the profile only for SSA, much like that of ovalbumin, gamma-globulin, and fibrinogen in Figure 2. The specific sensitivity of albumin to the aromatic sulfonic acids depends on its structure involving the bridges. Such reduction probably destroys specific interaction sites with the acids on albumin.

**Discussion**

Interaction between proteins and acids determining turbidity. The aromatic sulfonic acids and low concentrations of the chloroacetic acids give greater turbidity for proteins with high pI than for ones with low pI, except for albumin. A protein has side-chains with dissociable groups (acid groups to negative and basic groups to positive), non-dissociable polar groups, and non-polar groups. Under the turbidimetry conditions, the acid groups have no charge with pK_{a}' of 3.86 and 4.25 at 25 °C for Asp and Glu, respectively, and all of the basic groups have positive charges with pK_{a}' of 12.48, 10.53, and 6.00 for Arg, Lys, and His, respectively (15). When the acids are added to a protein solution, the protein with a higher pI has more anions of the acids adsorbed on its positive charges, producing greater turbidity than one with lower pI.

The chloroacetic acids in high concentrations give great turbidity for any protein, regardless of its pI, accompanying the change in the brightness of the precipitate, from light to dark, according to the increase in the acid concentration. Probably the light precipitate is rich in water and the dark one poor. Such increase is more pronounced for DCA than TCA. DCA is a weaker acid than TCA with pK_{a}' of 1.29 at 25 °C for DCA and 0.65 for TCA (15). Therefore, non-ionized acids (CCl_{2}HCOOH, CCl_{3}COOH) are possibly important in the turbidimetry-increasing process. Grimbly and Ntaiilicas (16) considered that the peptide groups of the protein backbone could provide hydrogen-bonding sites for non-ionized TCA because of the very large amount of TCA bound to milk proteins. However, the turbidity profiles of pepsin in Figure 2, with many —COOH side-groups, which are hydrated more readily than the peptide groups (17), are more reasonably explained by the mechanism given in Figure 7. The —COOH side-groups as well as the peptide groups of a protein can provide the hydrogen-bonding sites for the —COOH groups of non-ionized DCA and TCA. According to this mechanism, DCA and TCA in high concentrations effectively eliminate many water molecules hydrating to the protein, thereby converting the appearance of the precipitate from light to dark.

The aromatic sulfonic acids specifically give great turbidity for albumin and such specificity depends on the structure of albumin, especially the disulfide bridges. This indicates that there are specific sites on albumin that interact with the aromatic sulfonic anions. It is now recognized that albumin has high affinity for anions with hydrophobic groups (18, 19), by hydrophobic interaction as well as ionic interaction. Probably the specific sensitivity of albumin to the aromatic sulfonic acids depends on such high affinity of albumin for the anions with hydrophobic groups.

Utility of turbidimetry. As discussed above, at least three interactions are important in determining turbidity: (a) acid anions neutralizing positive charges on the protein, (b) hydrogen-bonding of the non-ionized chloroacetic acids with the protein, and (c) hydrophobic interaction of the aromatic sulfonic anions with albumin. The turbidity production possibly consists of several processes: (a) the acid lowers the solubility of the protein molecules by decreasing the positive charges on the protein and lowering the pH (—COO— → —COOH), (b) the insoluble protein molecules aggregate, and (c) the aggregate produces the turbidity. In addition, the turbidity is affected by particle size, i.e., the state of dispersion of the aggregate. Thus the turbidimetry is the result of many interactions between acid and protein and processes of turbidity production.
Turbidity varies appreciably with various factors: the species of acid and protein, the concentrations of the acid, temperature, and duration of standing after the acid is added.

Therefore, it is hard to expect either high reproducibility or quantitativity in estimating proteins by turbidimetry. It should be used only as a qualitative technique, especially for confirming proteinuria in which albumin can be expected to be a major component, by use of SSA or other aromatic sulfonic acids at temperatures near 20 °C, rather than at 0 or 37 °C (Figure 1). According to our findings, it is possible that suitable turbidimetry for confirming proteinuria with or without albumin, e.g., low-molecular-mass proteinuria, can be effected by the combined use of DCA and an aromatic acid having a pHₐₐ many lower than that of DCA, e.g., SSA and DCA.

References