Heat Denaturation of Blood Serum Proteins Measured in Saturated Sodium Chloride[1]

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Heat denatured proteins in bovine serum were precipitated by saturation with sodium chloride, filtered, and the undenatured components in the filtrates measured turbidimetrically at 620 mμ. Denaturation followed a logarithmic relationship to temperature and showed only a slight response to time at the temperatures studied. The albumin, post albumin, transferrin, and globulin fractions showed different individual denaturation responses to heat as monitored by electrophoretic analysis.

Investigations into the preparation of a food grade product from blood proteins have required a study of the effect of heat denaturation on these blood serum components. Tekman and Öner (1966) demonstrated that blood serum samples heated for 15 min at 55° C showed electrophoretic patterns similar to that of unheated serum, samples heated at 65° C showed decreased albumin mobility and no fractionation of globulins, and samples heated at 75° C showed no migration of any protein fraction. Further, the structure of bovine serum albumin is altered by heat (Maurer, 1959), resulting in a loss of immunological reaction with bovine serum albumin antisera while maintaining the solubility characteristics of native bovine serum albumin. Van der Scheer *et al.* (1941) described an interaction between albumin and globulins during heat treatment of blood serum which resulted in the development of a colloidal aggregate.

The turbidimetric procedure of Harland and Ashworth (1947), for the estimation of whey proteins in milk, appeared adaptable for the estimation of undenatured proteins in heat treated blood serum. Preliminary investigations indicated that denatured blood proteins are precipitable by NaCl similar to the milk proteins, and that this method is more rapid than published procedures for monitoring blood serum protein denaturation. The purpose of this investigation was to develop this rapid procedure for quantitatively measuring the heat denaturation of blood serum proteins and to determine whether the individual serum protein fractions respond uniformly to heat treatments.

MATERIALS AND METHODS

Preparation of Blood Serum. Fresh whole blood was obtained from steers at the time of slaughter. The blood was allowed to clot and the serum exudate was cooled, centrifuged, and the clear top layer removed for use in this experiment. Blood serum prepared in this manner was stored at 50 C, and in all instances was used within 48 hr of preparation. Throughout this work, bovine serum diluted 1 to 10 (referred to as 10% serum) with physiological saline was used to ensure against gelation of proteins during heating.

Preparation of Standard Curve. A standard curve expressing turbidity as a function of protein concentration was prepared by mixing, in various proportions, sera containing high and low amounts of protein. The low protein solution was prepared by heating 100 ml of 10% serum in a boiling water bath for 45 min with constant agitation. Another 100 ml portion of the 10% serum was not heated and was used for the high protein serum. Forty grams of NaCl (certified ACS, Fisher Scientific Company) was added to each protein solution, and the resulting mixture filtered through Whatman No. 40 filter paper to remove denatured protein. The protein concentrations in the low and high protein sera were determined according to the method of Lowry *et al.* (1951), using pure bovine serum albumin as the standard. Aliquots of the NaCl saturated filtrates were combined to prepare serial protein dilutions. One ml of each combination was diluted with 9 ml of saturated aqueous NaCl solution, mixed thoroughly, and 0.10 ml of 10% HCl added to develop turbidity in the protein. Turbidity was measured after 10 min on a Coleman Junior spectrophotometer at 620 mμ. This was established experimentally as the optimum wavelength for determinations in blood serum.

Heat Treatment of Blood Sera. Duplicate 10% serum samples (10 ml) were heated in a water bath at 60°, 70°, and 80° C for 30 min in 16 × 150 mm screw-cap test tubes. The samples were constantly agitated with a reciprocating shaker operating at 200 cycles per min. Timing was started when the tube contents reached the desired temperature. The tubes were cooled immediately with tap water at the end of the heating period.

Denaturation Calculation. The samples of heated sera and an unheated 10% serum sample were saturated with 4 g of NaCl per 10 ml, similar to the Harland and Ashworth (1947) technique. The NaCl saturated solutions were filtered through Whatman No. 40 filter paper, and 1 ml aliquots were diluted and treated with HCl to develop turbidity, as in the preparation of the standard curve. Turbidity readings were compared to the standard curve to estimate the amount of protein in each. Protein removed by heating and subsequent NaCl saturation was determined by difference, and is reported as a percentage of the protein content of the unheated serum to provide our figures for percent denaturation.

Electrophoretic Analysis. Disc electrophoresis was used to determine the response of the individual protein components to heat treatment and NaCl precipitation. The electrophoretic procedures developed by Rausch *et al.* (1965) were used for this work. A current of 2.5 mA per tube was maintained until the tracking dye was 1/4 in. from the bottom of the tubes.

Protein bands were stained with Amido Black 10B, and densimetric measurements of the stained bands were made with a Photovolt Densicord at the linear response setting. Protein fractions were identified from standard bovine serum protein components, and by comparison to the published in-

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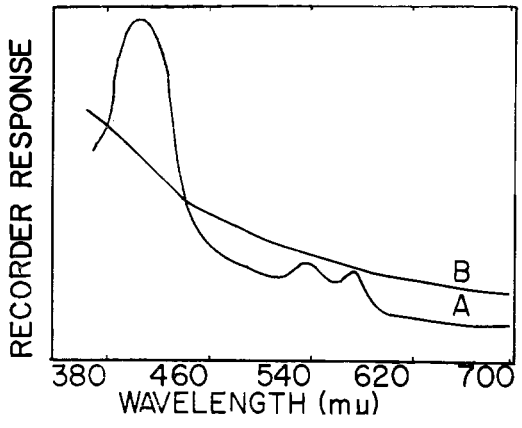

Figure 1. Spectrophotometric scans of blood serum (A) and serum with developed turbidity (B)

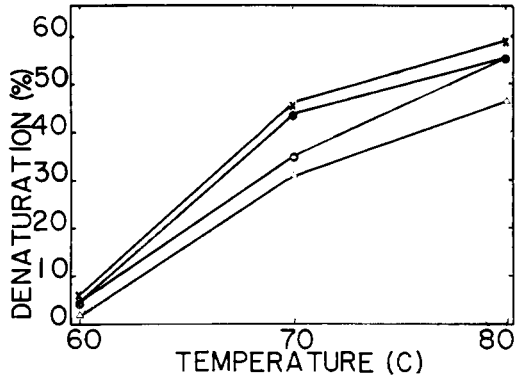

Figure 2. Effect of temperature on denaturation of blood serum protein at 5 (△), 10 (○), 20 (●), and 30 (×) min heating time

formation of Ornstein (1964). Quantitative estimates of the individual protein components were obtained by directly weighing the chart paper cut out under the individual peaks in the densimetric tracings.

RESULTS AND DISCUSSION

Preliminary experiments investigating the applicability of the Harland-Ashworth turbidimetric procedure to blood serum protein determinations indicated a good turbidimetric response to the concentration of the blood proteins. However, the wavelength (420 mμ) established for the Harland-Ashworth procedure is within the Soret band for hemoglobin and therefore interference by this band with turbidimetric measurements would be expected.

To establish the best wavelength for reading turbidity, a Beckman Model DB spectrophotometer was used to scan the absorption spectrum of unheated serum. Whole serum was diluted (1 to 10) to maintain absorbancy peaks in a readable range on the recorder scale. The absorption spectrum presented in Figure 1 is typical of previously reported results (Oser, 1965) for blood serum exhibiting light absorption maxima at 420, 535, and 570 mμ. The absorbance response is elevated significantly in the region of the lower wavelengths. Therefore, diluted blood serum, with turbidity developed according to Harland-Ashworth (1947), was scanned to locate the point of optimum turbidimetric response (Figure 1). The overall absorbance represented in the turbid sample is less than that in the unheated serum because of the higher dilution (1 to 100) in the turbid sample. While more response to turbidity was observed below 600 mμ, the greatest contribution of any contaminating hemoglobin also occurred below 600

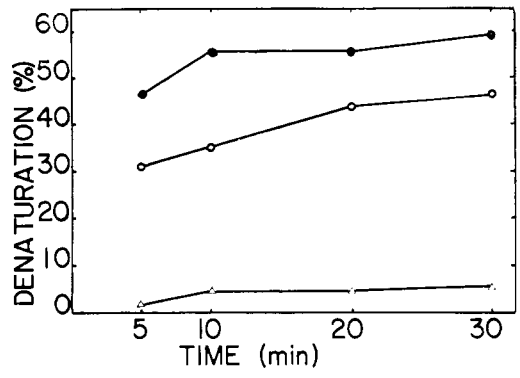

Figure 3. Effect of time on denaturation on blood serum proteins at 60° (△), 70° (○), and 80° (●) C

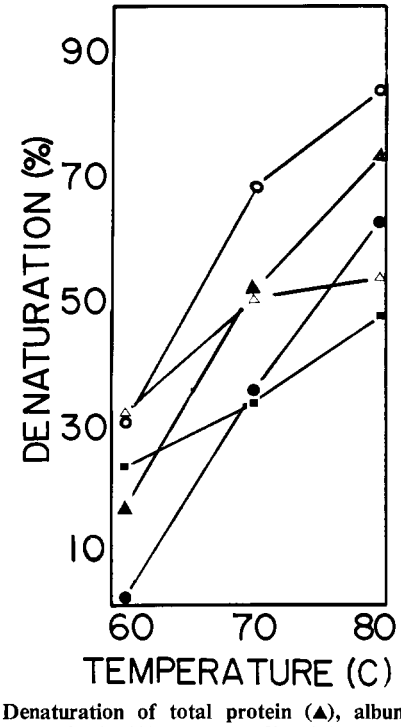

Figure 4. Denaturation of total protein (▲), albumin (○), post albumin (■), transferrin (△), and globulins (●) as measured densimetrically from electrophoretic analysis

mμ. Therefore, we concluded that 620 mμ would be used for turbidimetric measurements in these experiments.

A standard curve was prepared at 620 mμ to express turbidity as a function of protein concentration. Protein concentration ranged from 0.10 to 0.95 mg per ml. The linear equation expressing optical density as a function of protein concentration within this range was calculated as:

$$y = 0.0143 + 0.1102x$$

The linear regression coefficient was highly significant ($P < 0.001$). The calculated y-intercept value (0.0143) is slightly above 0, but it must be remembered that responses to protein concentrations were not determined below 0.10 mg/ml. Thus, the curve indicated sufficient response from the modified method for good sensitivity over an adequate range of protein concentrations.

The modified turbidimetric method was used to measure the extent of protein denaturation in blood sera heated at several temperatures for different times. The results expressing serum protein denaturation as a function of temperature and time in Figures 2 and 3 were the average of two independent

trials. The data in Figure 2 show that denaturation increases directly with temperature. The coincidence of the two points at heating times of 10 and 20 min probably was the result of experimental error.

Denaturation response to time (Figure 3) was less significant than expected from denaturation patterns of milk serum proteins (Dill *et al.*, 1964). There is a proportional increase in denaturation with increases in time, though apparently a given level of denaturation is much more a function of temperature than of time. This type of response might result from differences in the sensitivity of individual protein moieties to heat denaturation.

To determine the distribution of protein types after heat treatment, NaCl filtrates were subjected to polyacrylamide gel electrophoresis. The results for denaturation presented in Figure 4 were obtained by calculation of the percentage change in densimetric tracings of individual components as a result of heat treatment. These results are the average of four trials, and while there were slight differences between trials, the patterns within a trial were consistent. The result for denaturation of the total (pooled) serum proteins determined densimetrically shows a relationship to heat treatment which is consistent with the data presented in Figure 2, though the amount of denaturation is consistently about 10% higher than data generated by turbidimetric measurement. Albumin denaturation is consistently higher than the pooled value, and denaturation of globulins is consistently lower, but parallel to both the albumin and the pooled data. By comparison, the results for post-albumin and transferrin, as defined by Ornstein (1964), indicate a greater sensitivity to heat than the pooled value at 60° C, but a lower response to increments of heat treatment within the range of temperatures studied here. The albumin and globulin denaturation agrees closely to the pooled value, probably due to their concentrations in blood serum. The protein components in our raw sera showed the following means: albumin 34.1%, post albumin 12.6%, transferrin 22.3%, and globulins 30.87%. Thus, the combined albumin and globulin fractions represented about 65% of the total serum proteins.

The proteins monitored electrophoretically and summarized in Figure 4 actually represent a NaCl-soluble fraction. We assume that molecular changes brought about by heat denaturation are irreversible, and are such as to make the denatured molecules unstable in the presence of saturated NaCl. We interpret the results represented in Figure 4 as suggesting that certain blood serum protein components are less sensitive to the effects of heat. However, the possibility remains that significant changes do occur, but are not of a nature to result in a loss of stability in the presence of saturated NaCl.

CONCLUSIONS

The NaCl precipitation technique offers a useful method for monitoring the heat denaturation of blood serum proteins. Results with the technique are obtained rapidly, and are in agreement with results by other methods used to study the response of serum proteins to heat treatment. The distribution of proteins remaining in suspension after NaCl saturation is not constant over a wide range of heat treatments, presumably due to differences in the denaturation rates among the several protein components.

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