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Abstract

2-Mercaptoethanol increases the optical density of assay solutions at wavelengths between 280 to 400 nm, and therefore interferes with the measurement of protein concentration by the microbiuret method. Protein concentration can be determined in the presence of 2-mercaptoethanol up to 6 mM by modification of the method as follows: after the precipitation of protein by trichloroacetic acid in the presence of deoxycholate, the precipitate is resolubilized with NaOH solution. Dithiothreitol interfered with the protein determinations could by made in the presence of 4 mM of dithiothreitol with the modified microbiuret method. This modified method is time-saving and more reliable than other methods for protein determination, such as Lowry's method, in the presence of sulfhydryl reagents.

KEYWORDS: microbiuret method, sulfhydryl reagent, protein determination

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MODULATION OF OPTICAL DENSITY BY SULFHYDRYL REAGENTS IN MICROBIURET METHOD: A MODIFIED METHOD FOR PROTEIN DETERMINATION IN THE PRESENCE OF SULFHYDRYL REAGENTS

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Abstract. 2-Mercaptoethanol increases the optical density of assay solutions at wavelengths between 280 to 400 nm, and therefore interferes with the measurement of protein concentration by the microbiuret method. Protein concentration can be determined in the presence of 2-mercaptoethanol up to 6 mM by modification of the method as follows: after the precipitation of protein by trichloroacetic acid in the presence of deoxycholate, the precipitate is resolubilized with NaOH solution. Dithiothreitol interfered with the protein determination even more than 2-mercaptoethanol, but determinations could be made in the presence of 4 mM of dithiothreitol with the modified microbiuret method. This modified method is time- saving and more reliable than other methods for protein determination, such as Lowry's method, in the presence of sulfhydryl reagents.

Key words: microbiuret method, sulfhydryl reagent, protein determination.

The microbiuret method for protein determination, introduced by Itzaki and Gill (1), is based on the measurement of the ultraviolet light absorption by the protein and copper complex formed in a strongly alkaline copper sulfate solution. This method is widely used, because it is ten times more sensitive than the ordinary Biuret method (1), it is time-saving since color development is complete in five minutes and stable for 2.5 h. (1), and it is nonspecific for the type of protein, *i.e.*, independent of amino acid composition (1). Very few substances interfere with protein determination by the biuret method in comparison with other methods, such as that of Lowry (2-4).

Sulfhydryl reagents, such as 2-mercaptoetanol and dithiothreitol, are commonly used to protect sulfhydryl groups of proteins. The influense of these reagents on optical density measured by the microbiuret method have not been reported.

In this report we show that 2-mercaptoethanol and dithiothreitol strongly influence light absorption measured by the microbiuret method, and we present a modified microbiuret method for determining protein concentration in the pres-

ence of these reagents.

MATERIALS AND METHODS

All reagents used were of analytical grade. Bovine serum albumin (BSA) was purchased from Katayama Chemical Industries, Ltd., Osaka, Japan, and 2-mercaptoethanol and dithiothreitol from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

The microbiuret reagent contained 0.21 % $CuSO_4 \cdot 5H_2O$ in 30 % NaOH. BSA and sulfhydryl reagents were dissolved in deionized water. Light absorption was measured 10 min after the addition of the microbiuret reagent to the sample solutions at room temperature. Photometry was performed with a Shimazu UV210-A digital double beam spectrophotometer.

We modified the microbiuret of Itzaki and Gill as follows: 100 μl of 0.4 % Na-deoxycholate and 1 ml of 24 % trichloroacetic acid were added to 2 ml of sample solution, and the reaction mixture was vigorously shaken for 5 sec and centrifuged at 3,500 rpm for 10 min in a Sakuma centrifuge, model 90-22. The supernatant was aspirated carefully and the precipitate was redissolved with 2 ml of 0.89 N NaOH. Coloration and measurement of the optical density were performed as described above. Standard BSA was dissolved with 0.89 N NaOH and processed in the same way.

RESULTS

The presence of 1.5 mM 2-mercaptoethanol in the sample solution increased the optical density (O.D.) at 310 nm by about 0.15 unit at each protein concentration as shown in Fig. 1. This increase was independent of protein concentration and was observed equally in the absence of protein.

Fig. 2 shows that 2-mercaptoethanol changed the optical density significantly. From 1 mM to 3.5 mM of 2-mercaptoethanol, the optical density of the reaction

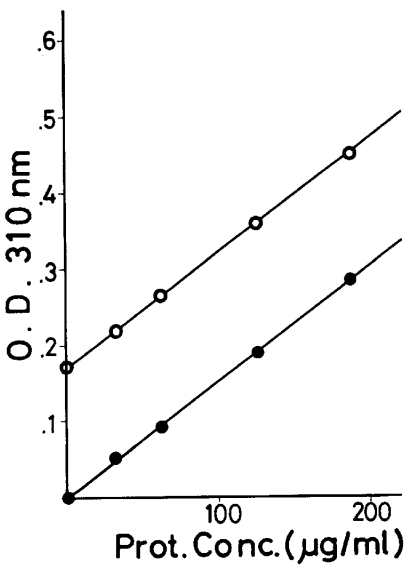

Fig. 1. Effect of 2-mercaptoethanol on protein determination by the unmodified microbiuret method. Open circles (○), 1.5 mM 2-mercaptoethanol; filled circles (●), no 2-mercaptoethanol in the 2 ml sample solution.

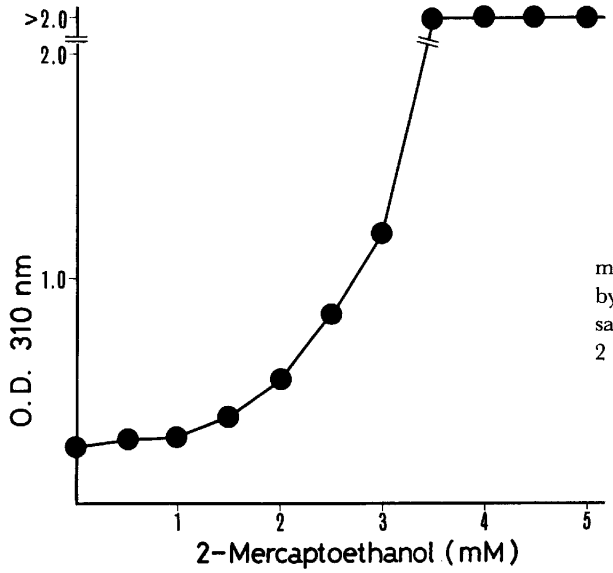

Fig. 2. Effect of the amount of 2-mercaptoethanol on protein determination by the original microbiuret method. Each sample contains 125 μg/ml of BSA in the 2 ml sample solution.

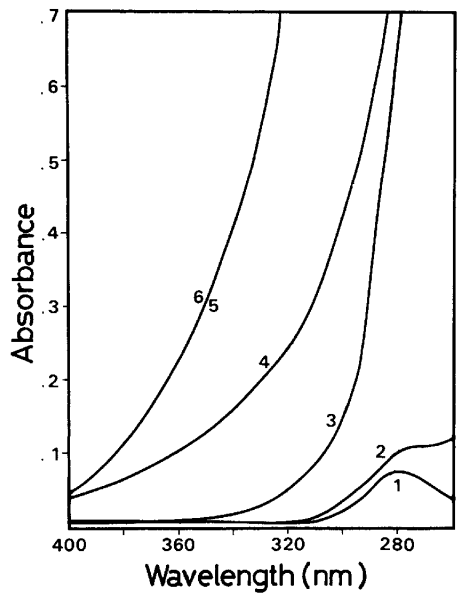

Fig. 3. Ultraviolet absorption spectra of reaction mixtures with or without 2-mercaptoethanol.
1. 125 μg/ml BSA
2. BSA + 1.5 mM 2-mercaptoethanol
3. Microbiuret reagent
4. BSA + Microbiuret reagent
5. Microbiuret reagent + 2-mercaptoethanol
6. Microbiuret reagent + 2-mercaptoethanol + BSA
(2 ml sample solutions)

mixture sharply increased. Samples containing 2.5 mM 2-mercaptoethanol became turbid.

Other sulfhydryl reagents such as dithiothreitol, L-cysteine and reduced glutathione showed stronger effects (data not shown). Ethanol had no effect on the microbiulet method up to a concentration of 10 mM (data not shown).

The increase in the optical density of reaction mixtures in the presence of

2-mercaptoethanol was observed in the absence of protein (Fig. 3), which demonstrates that the increase in the optical density is due to the interaction of 2-mercaptoethanol and the microbiuret reagent.

Protein determination by the modified microbiuret method was unaffected by 2-mercaptoethanol even at 6 mM (Fig. 4).

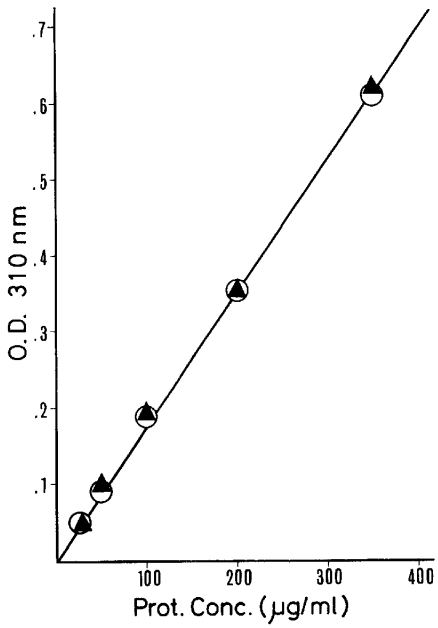

Fig. 4. Removal of interference by 2-mercaptoethanol through modification of the microbiuret procedure. Circles (○), without 2-mercaptoethanol in the unmodified procedure; triangles (▲), with 6 mM 2-mercaptoethanol in the modified procedure.

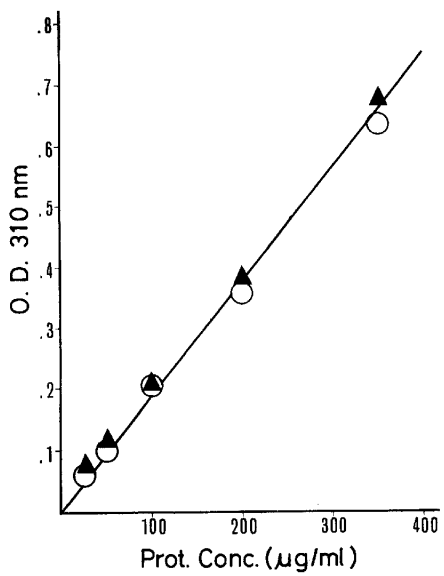

Fig. 5. Elimination of the dithiothreitol effect by the modified procedure; Open circles (○), without dithiothreitol in the unmodified procedure; triangles (▲), with 4 mM dithiothreitol in the modified procedure.

Dithiothreitol caused a much larger increase in the optical density as measured by the original microbiuret method than did 2-mercaptoethanol, but interference by dithiothreitol up to 4 mM was completely overcome by modification of the method as described in the present paper (see Fig. 5).

DISCUSSION

In the original microbiuret method, interference by 2-mercaptoethanol was independent of protein concentration (Fig. 1). Sample solutions containing the microbiuret reagent and 2-mercaptoethanol showed the same ultraviolet absorption spectra in the presence or absence of protein (Fig. 3). Ethanol did not affect the determination. These results indicated that the change in the optical density was dependent on the direct interaction between the microbiuret reagent and the sulfhydryl group.

In the modified method presented in this paper, proteins were precipitated with trichloroacetic acid, and interfering substances were eliminated in the supernatant. In the presence of deoxycholic acid, the previous report by Bensadoun and Weinstein (5).

As shown in Figs 4 and 5, the modified method resulted in a satisfactory protein determination in the presence of a high concentration of the sulfhydryl reagents.

After modification, the method remains time-saving and nonspecific for the type of protein.

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