A BIURET REAGENT COMPOSED OF COPPER SULFATE, SODIUM HYDROXIDE AND AMMONIA FOR THE DETERMINATION OF SERUM PROTEIN

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Routine procedure for the determination of serum protein has enjoyed a considerable simplification since Weichselbaum and others[1,2,3,4] introduced biuret reagents, which supplanted the time-consuming Kjeldahl's method. Recently Levin and Brauer[5] have reported another biuret reagent which was composed of copper sulfate, sodium hydroxide and ammonia. Its simple composition prompted us to examine its utility, and it was appreciated as one of the excellent solution for the determination of serum protein after its composition was modified so as to improve the final coloration. Our modification will be presented below.

METHOD

Reagent

1) Biuret reagent: Dissolve 1.7g. of crystalline copper sulfate ($CuSO_4 . 5H_2O$) in a small amount of water, and add 300ml. of the mixture of caustic ammonia (sp. g. 0.895) and water (1:1). Add to the solution thus produced 100 ml. of saturated aqueous solution of sodium hydroxide (approximately 20 N) slowly under constant stirring, and make to volume with water in a 500 ml. graduated cylinder. Preserve in a bottle plugged with a rubber stopper to prevent evaproration.

2) Aqueous solution of sodium sulfate (anhydrous, Na_2SO_4), 27.8g./dl. in concentration. Presserve in a bottle tightly stoppered and keep in an incubator adjusted to 37 C.

3) Standard protein solution: Determine the concentration of protein (Sg./dl.) of a serum sample of healthy person by Gunning-Arnold's nitrogen determinatoin[6], and use the serum in combination with its two-fold aqueous dilution as standard protein solution of Sg./dl. and S/2 g./dl.

Procedure

A. Determination of total serum protein.

Introduce aliquots of 0.1 ml. of blood serum, standard protein solution, its two-fold aqueous dilution and distilled water into test tubes A, B, C and D, respectively. Burette 4.0 ml. of biuret reagent to each, mix by inversion, allow to stand at room temperature for five minutes and read in a photo-electric colorimeter the absorbances a, b and c for the mixtures A, B and C at 560 mμ against the mixture D as reagent blank.

Plot a rectilinear line which passes the points (S, b) and (S/2, c) in association with a line whose ordinate is equal to a on a rectangular co-ordinate graph, with protein concentration as the ordinate, determine the point of intersection, and read its abscissa to obtain the

value for the unknown concentration of serum protein.

B. Determination of albumin to globulin ratio.

1) Mix in a test tube 0.5 ml. of the serum with 7.5 ml. of sodium sulfate solution, inverting two to three times with tightly stoppered rubber plug into the orifice of the tube, incubate at 37 C for three hours or more, mix again by inversion, and filter a moiety of the mixture through a filter paper Toyo-roshi No. 7 at room temperature of more than 20 C to obtain clear filtrate. The remainder of the mixture is preserved and designated S fluid.

2) Prepare a series of test tubes (A, B, C and D) as listed below, add 3.0 ml. of biuret reagent to each, mix by inversion with thumb at the orifices of the tubes, and allow the mixtures thus obtained to stand for five minutes to secure complete coloration.

Test tube	S fluid	Filtrate	Sodium sulfate solution
A	1.5 ml.		0.5 ml.
B	0.5 ml.		1.5 ml.
C		2.0 ml.	
D			2.0 ml.

3) Read at 560 mμ the absorbances a, b, c and d for the mixtures A, B, C and D, respectively, against the mixture D as reagent blank. Plot a rectilinear line which passes through the points (75%, a) and (25%, b) on a rectangular co-ordinate graph, with protein concentration as the abscissa against absorbance as the ordinate, regarding the mixtures A and B to have 75 and 25 per cent of the protein of the undiluted serum. Read the abscissa X of the point of intersection where this line crosses the line which is parallel to and by distance c apart from the abscissa. The value X represents the albumin fraction in per cent of the total serum protein, and the globulin fraction is given as (100–X). The serum albumin to grobulin ratio is accordingly calculated as x/(100–x).

The reliability of these procedures were checked as follows:–

(1) Detection of maximum absorbance: The absorption band of the serum protein colored by this biuret reagent was searched for over the whole range of visual spectrum (470–650 mμ) against reagent blank (2.0 ml. of water added by 4.0 ml. of biuret reagent) in Erma's photoelectric spectrophotometer with a cube cell of 10 mm. optical path. Aliquots of 0.1 ml. of serum sample and of their 1.5 and 3.0 fold aqueous dilution were mixed with two ml. of water and 4.0 ml. of biuret reagent.

(2) Obedience to Beer's law and reliability of the determination: The absorbance of the colored solution was read on serum samples in combination with their 1.5 and 3.0–fold aqueous dilutions, each in duplicate, in a photoelectric colorimeter with filter S 57 which has maximum transnission around 570 mμ and test tube cell of 7 mm. diameter, to observe whether or not the coloration obeys Beer's law as well as to examine if discrepancy between the duplicate determination exceeds 2 per cent. As for the coloration the procedure described in A was strictly followed.

(3) Collation to Kjeldahl azotometry: Serums were determined for total protein and albumin globulin ratio by this biuret procedure and azotometry to compare with each other. Gunning-Arnold's Kjeldahl technique[6] adapted to photoelectric colorimetry was employed for the azotometry, and the N determination (total N–NPN mg./dl.) multiplied by 0.00625 were regarded as protein concentration (g./dl.)

Results and Discussion

Levin and Brauer[5] specified to mix 2.0 ml. of their reagent with 3.0 ml. of the protein solution ranging in concentration from 0.005 to 0.125 g./dl., but in our experience this failed to develop a perfect coloration because of the defi-

ciency of copper ion, and occasionally resulted in turbidity before the colored solution was subjected to a colorimeter. Limpid fully developed coloration was secured when 4.0 ml. of our biuret reagent whose copper content was modified to duplicate that of Levin-Brauer's was utilized against 2.0 ml. of the protein solution of 0.05 to 0.5 g./dl.

Our reagent is dark purplish-blue with maximum light absorption at 630 mμ, as clearly shown in Figure 1, and yields a purple color when mixed with protein,

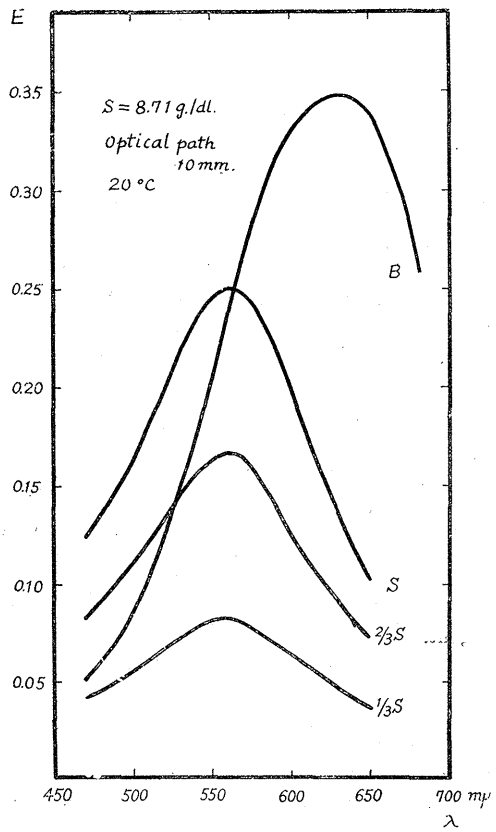

Fig. 1. Light absorption of protein solutions
(S, $\frac{2}{3}$S and $\frac{1}{3}$S ; S denotes concentration of serum protein)
and reagent blank (B). E: absorbance, λ: wave length.

displaying a single absorption peak at 560 mμ. The color attains perfect development five minutes following the addition of the reagent and remains stable for at least ten minutes thereafter. Figure 2 discloses its obedience to Beer's law at the wave lengths around the absorption peak. The absorbance exhibits an appreciable increase while a dilute protein solution (short of 0.1 g./dl. in concentra-

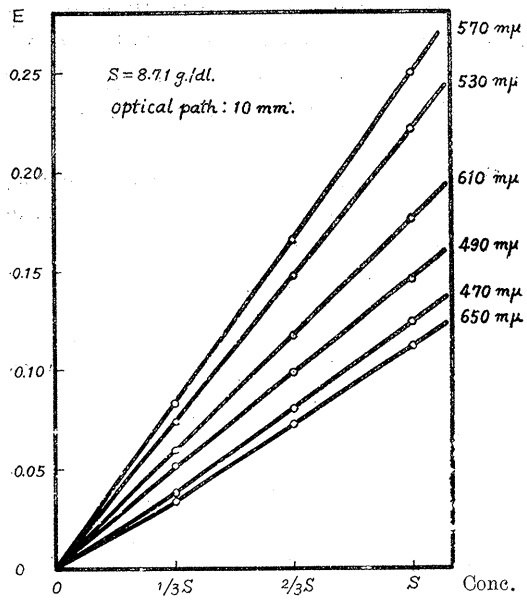

Fig. 2. Obedience to Beer's law

tion) is allowed to stand fifty minutes or more after its treatment with biuret reagent. In such a case a rise of as much as 16 per cent in absorbance is encountered during the period of fifteen to thirty minutes owing to the slight turbidity which develops more than fifteen minutes later, whereas little change is noted in case protein solution is concentrated. Temperature has little effect upon the coloration, and the absorbance remains constant over the range of room temperature from 15 to 30 C. However, reading from a calibration curve which was prepared beforehand with regard to a solution whose protein concentration was known is not recommended, since slight diversity in coloration may arise from day to day with obscure cause.

Our reagent enables the fractionation of serum protein into albumin and globulin when it is applied to Milne's salting out method[7] in which 27.8 g./dl. aqueous solution of sodium sulfate is used.

Tables I and II disclose good agreement of our procedure with the azotometry in the determination of total protein in serum as well as in its fractionation. The divergence in duplicate determination remains within two per cent of the estimation.

The reagent is only disadvandageous in an offensive and irritating smell which impedes pipetting.

SUMMARY AND CONCLUSION

TABLE I

Comparison of biuret method with Gunning-Arnold's N-determination

Biuret method, in duplicate		N-determination
5.90g./dl.	5.99g./dl.	5.91g./dl.
6.59	6.63	6.57
6.85	6.89	6.95
7.21	7.24	7.28
7.45	7.61	7.73
7.63	7.63	7.78
7.67	7.65	7.59
7.77	7.78	7.61
8.46	8.60	8.70
9.04	9.04	9.38

TABLE II

Comparison of biuret method with Gunning-Arnold's N-determination

Total protein g./dl.		Albumin per cent of total protein		Globulin per cent of total protein		Serum A/G ratio	
Biuret	N-determ.	Biuret	N-determ.	Biuret	N-determ.	Biuret	N-determ.
9.8	9.24	8.0	6.0	92.0	94.0	0.09	0.06
8.1	8.09	34.0	34.1	66.0	65.9	0.52	0.52
7.2	7.20	35.7	36.0	64.3	64.0	0.56	0.56
6.7	6.79	41.7	40.5	58.3	59.5	0.71	0.68
7.5	7.60	42.2	41.5	57.8	58.5	0.73	0.71
7.5	7.56	47.2	47.1	52.8	52.9	0.89	0.89
7.2	7.44	50.5	50.3	49.5	49.7	1.02	1.01
4.1	3.81	28.0	23.0	72.0	77.0	0.39	0.30
6.0	6.01	41.6	41.6	58.4	58.4	0.71	0.71
7.2	7.39	47.3	48.2	52.7	51.8	0.90	0.93
7.1	7.13	51.0	50.2	49.0	49.8	1.04	1.01
7.0	7.09	52.7	51.9	47.3	48.1	1.12	1.08
8.8	8.66	59.0	56.0	41.0	44.0	1.44	1.27
7.3	7.33	59.2	58.6	40.8	41.4	1.45	1.43

Levin-Brauer's biuret reagent consisted of copper sulfate, sodium hydroxide and ammonia was modified in composition to secure stable coloration when mixed with protein solution. An aliquot of 0.1 ml. of blood serum was diluted with 2.0 ml. of distilled water, and colorized with 4.0 ml. of the improved biuret reagent. The absorbance was read, five to fifteen minutes after mixing, in a photoelectric colorimeter with a filter whose maximum transmission was at 570 mμ to obtain the concentration of total serum protein. For the determination of albumin and globulin in serum the procedure was coupled with the sodium sulfate salting out method of Milne. The coloration attained to maximum stability with this reagent within shorter period of time than with Weichselbaum's. The results were in satisfactory accordance with those of azotometry.

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