

# DETERMINATION OF SERUM PROTEINS BY MEANS OF THE BIURET REACTION

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In the course of an investigation of the biochemical changes following experimental liver injury we felt the need of a simple, rapid, and accurate method for determining the protein fractions in small amounts of serum. Among the simpler procedures known, the biuret reaction seemed to offer the most encouraging possibilities.

Variations and improvements in the application of the biuret reaction to clinical chemistry can be traced in the works of Autenrieth (1), Hiller (2), Fine (3), Kingsley (4), and Robinson and Hogden (5). Kingsley (6) simplified the technique by adding serum directly to a "one piece" reagent. Efforts have been made to increase the stability of such biuret reagents with ethylene glycol (7), tartrate (8), and citrate (9).<sup>1</sup>

We began our investigation with Kingsley's (6) method and report briefly on the two main difficulties encountered in its use. The first is that the total protein (TP) reagent and, to a lesser extent, the albumin (ALB) reagent are not sufficiently stable. The length of time they remain so depends upon the technique of their preparation. One consequence of this variable stability is a difficulty in duplicating calibration curves with different lots of reagent. Errors may arise when results with a new reagent are read from an old calibration curve. Serious errors occur if a reagent is used after the separation of any black deposit gives evidence of deterioration.

A second difficulty has been that total protein estimations made with the TP reagent and read, as prescribed, from calibration curves prepared with the ALB reagent have tended to be too low. Recorded in Table I are the results of a number of analyses in which Kingsley's biuret procedure has been compared with the Kjeldahl method<sup>2</sup> on both normal and ab-

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<sup>1</sup> We have had access only to an abstract of this paper. Even with 10 times the amount of citrate there stated the reagent has been found unstable.

<sup>2</sup> Kjeldahl digestion with copper selenite catalyst (10) heated 15 to 20 minutes after clearing. Distillation into boric acid and titration with standard acid (11); protein = nitrogen (corrected for non-protein nitrogen)  $\times$  6.25. Values so obtained were 99+ per cent of the results with a digestion time of 3 hours or longer.

normal human and dog sera. It will be noted that, in such a comparison, the results for total protein by Kingsley's method are invariably and considerably low, while the results for albumin are in reasonably good agreement. Kingsley (12) reported low values for total protein by his method in patients suffering from chronic liver disease, but good agreement with the Kjeldahl method in normal individuals or persons suffering from a variety of other diseases. We have noted the discrepancy in any serum when a result for total protein with the TP reagent is read from a calibration curve prepared with the ALB reagent. The error, it is felt, must be

TABLE I

*Results of Comparison of Analyses by Kingsley and Kjeldahl Methods*

The values are measured in gm. per cent.

Serum		Diagnosis	Total protein			Albumin	
			Kjeldahl nitrogen	Kingsley biuret		Kjeldahl nitrogen	Kingsley biuret
				Total protein reagent	Albumin reagent		
Human	A. G.	Normal	7.49	7.10	7.45		
	C. B.	"	7.01	6.60	7.10		
	B. B.	"	7.04	6.50	7.10		
	J. D.	"	6.70	6.30	6.65		
	McK.	Lupus erythematosus	7.38	6.30		3.45	3.04
	M. S.	Cirrhosis	6.00	5.00		3.17	3.07
Dog No.	1	Normal	6.32	5.92			
	2	"	6.55	6.05		3.68	3.36
	5	"	6.36	6.17		3.94	4.00
	14	"	5.95	5.30			
	15	"	6.34	5.95			
	1	CCl <sub>4</sub> liver injury	5.40	5.00	5.53	3.26	3.52
	10	" " "	6.32	5.92	6.30	3.38	3.22

attributed to differences in behavior of the two reagents, prepared and stored at different strengths, one diluted to the strength of the other only at the time of analysis. Note, for example, that in Table I, sixth column, where total protein is determined by adding diluted serum to the ALB reagent, agreement with the Kjeldahl determination is satisfactory.

Our experience with Mehl's (7) method has been more limited. The reagent is stable but not easily duplicated, especially if the glycol contains any reducing substances as impurity. Certain minor disadvantages of the method are not encountered in the procedures which follow.

Weichselbaum (8) has described a biuret reagent stabilized with sodium potassium tartrate and potassium iodide. It is characterized by its high

content of copper and low concentration of alkali. When a biuret reaction is developed with protein, the change appears to be simply an intensification of the blue color of the reagent, though spectrophotometric analysis reveals the presence of the reddish violet complex with an absorption maximum at about 545  $m\mu$ . In a foot-note, this author describes a second, "dilute" reagent as having the advantage of added sensitivity when the analyst is using a spectrophotometer or photoelectric colorimeter. This reagent has a fifth as much copper, and the reddish violet color developed upon addition of protein is clearly apparent.

It has been our experience that the biuret reagents of Weichselbaum possess the advantages claimed by their originator with respect to stability and optical clarity when mixed with clear sera. Differences between the two reagents, objections to the first, and a paucity of information concerning the behavior of either led us, however, to reinvestigate in some detail the factors affecting optimal sensitivity, stability, and practical usefulness of biuret reagents stabilized with tartrate. Our experiments, which have led to certain modifications in the reagent and in its application to the determination of protein fractions in serum or plasma, are described in the following section.

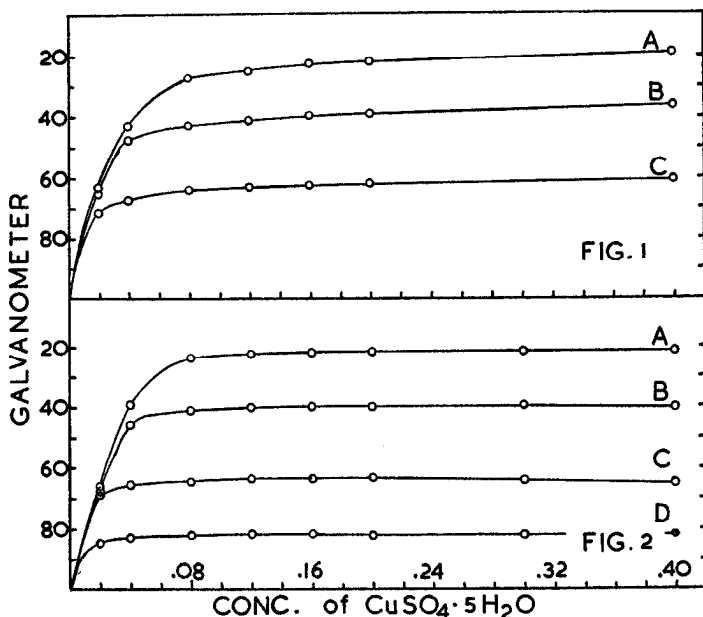
#### *Study of Biuret Reagents Stabilized with Tartrate*

*Concentration of Copper*—The concentration of cupric sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) in the final biuret reaction mixtures of different investigators has varied from about 0.06 to 0.75 gm. per 100 ml., most commonly in the case of serum protein estimations, from 0.1 to 0.2 per cent. Mehl has related the optical density of reaction mixtures to copper concentration for a reagent stabilized with ethylene glycol. Comparable data for a biuret reagent containing tartrate are reported in Figs. 1 and 2.

It will be noted in both Figs. 1 and 2 that, at the protein levels stated, color development increases with increasing concentrations of copper sulfate, rapidly at first, then more slowly, and finally (Fig. 1) reaches a stage in which further additions of copper effect only a very slight, uniform, and linear increase in optical density of the copper-protein complex. This last rise in the curve has been found to result from the change in tartrate to copper ratio, because (Fig. 2), when this ratio is held constant, the curve becomes essentially horizontal over a wide range of copper concentrations. It will be noted that at protein levels up to 0.04 per cent (4 per cent in the undiluted serum) a reagent yielding a final copper sulfate concentration of 0.06 per cent would be adequate. For sera containing about 7 per cent protein, the reaction mixture should contain not less than 0.09 per cent copper sulfate and, for levels up to 12 per cent protein, a concentration of 0.12 per cent is desirable. With the serum dilution used,

an increase in copper above this level makes no apparent contribution to the biuret reaction. Its chief effect is the somewhat undesirable one of increasing the optical density of the reagent "blank."

*Concentration of Sodium Hydroxide*—The importance of sodium hydroxide in the biuret reaction has long been recognized and was studied in some detail by Rising and Johnson (13). Before the development of stabilized reagents, the biuret reaction was generally carried out in a



FIGS. 1 AND 2. Effect of copper sulfate concentration upon color development in biuret reaction mixtures containing 2.4 per cent sodium hydroxide and protein concentrations of Curve A, 0.127 per cent; Curve B, 0.077 per cent; Curve C, 0.039 per cent, and Curve D, 0.016 per cent. In Fig. 1, the sodium potassium tartrate concentration is constant at 0.48 per cent. In Fig. 2, the tartrate concentration is in each case 3.5 times that of the copper sulfate present.

medium containing about 3 per cent alkali. It was necessary to separate a precipitate of cupric hydroxide before color comparisons could be made. Kingsley (6) found that very high concentrations of alkali (12 to 17 per cent) prevented cupric hydroxide precipitation and by this means obtained fairly stable "one piece" biuret reagents. With the introduction of reagents containing glycol or tartrate, the need for such high levels of sodium hydroxide was obviated. Mehl obtained best results with 2 to 4 per cent alkali in the presence of glycol. Weichselbaum concluded that 0.4 per cent sodium hydroxide was optimal for reagents containing tartrate.

We have investigated the effect of alkali concentration in a reaction mixture containing 0.12 per cent copper sulfate and 0.48 per cent sodium potassium tartrate and observed the color development shown in Fig. 3.

It will be noted that with 0.2 per cent alkali color development is poor, but that from a minimum of 0.4 per cent to about 7 per cent the results

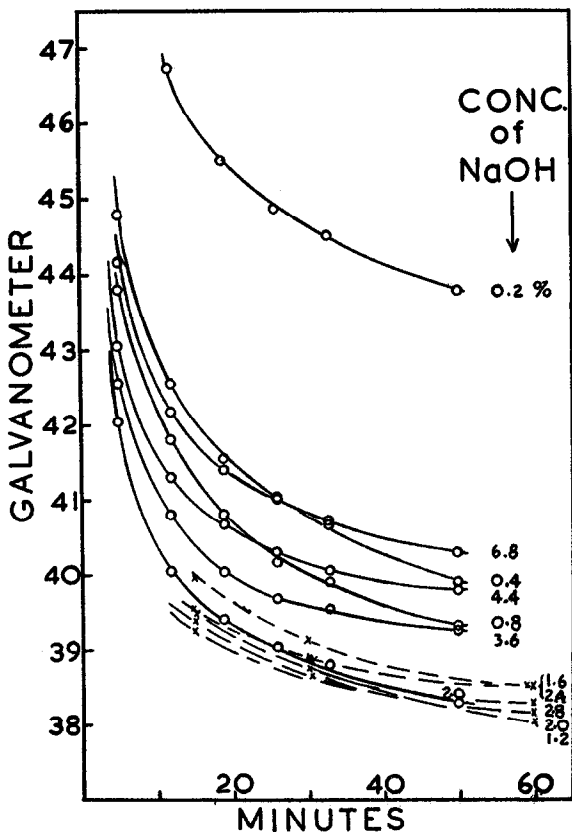


FIG. 3. The effect of sodium hydroxide upon color development in biuret mixtures containing 0.12 per cent copper sulfate, 0.48 per cent sodium potassium tartrate, and 0.077 per cent protein.

are not markedly different. With the final copper sulfate and tartrate concentrations here employed, it would appear that optimal biuret color development occurs with about 1.2 to 2.8 per cent alkali. It has been observed also that an increase in copper concentration tends to shift this optimal alkalinity toward higher values. From the shape of the curves shown it can be noted that the higher alkali concentrations favor a somewhat more rapid development of maximal color.

With low concentrations of alkali, a reaction mixture will remain clear for several days. When levels above about 2 per cent are used, the mixture may show, but only after 12 to 24 hours, a slight flocculent coagulum. This precipitation, which occurs when alkali alone is added to serum, has no significance for readings made at 30 minutes.

*Effects of Tartrate*—The use of sodium potassium tartrate as a stabilizing agent in the copper-containing reagents for the determination of reducing sugars has been known for many years. Shaffer and Somogyi (14) found that an amount of tartrate equal to about 3 times the weight of copper sulfate was most satisfactory in their reagent which contained approximately 3 per cent alkali. Weichselbaum employs triple amounts of tartrate in both of the low alkali biuret reagents which he has described.

We confirm the observation that a minimal 3:1 ratio of tartrate to copper sulfate is necessary if a biuret reagent containing moderate amounts of alkali is to keep longer than a few weeks. Having established a minimal copper sulfate concentration of about 0.1 per cent, it follows that the reagent must contain 0.3 per cent or more of sodium potassium tartrate. The addition of such amounts of tartrate causes a very slight retardation of, but a rather distinct diminution in the degree of, color development when protein is added to the reagent (Fig. 4). The presence of tartrate does make it advisable to wait somewhat longer before recording the optical density of the reaction mixture.

Weichselbaum felt it necessary to carry out the reaction in a warm water bath. In Fig. 4 there is also recorded, with our modified biuret reagent, the effect of developing and reading the color reaction at different temperatures. It will be noted that the effect is very slight after 30 minutes, amounting to about 1 scale division for every 10° rise in temperature. It would seem quite satisfactory therefore to develop the reaction at room temperature, within, say, a 5° range. This avoids entirely the complication of heating and, if the color intensity is read at 30 minutes, the changes are so slow that strict timing is not essential.

The inhibiting effect of tartrate is illustrated further in Fig. 5. Here we record, for different concentrations of copper sulfate and a constant amount of sodium hydroxide, the optical density obtained after 30 minutes in the presence of increasing ratios of sodium potassium tartrate to copper sulfate. It will be noted, first, that the greater the concentration of tartrate, for any fixed level of copper, the less the color development when protein is added to that reagent. A comparison of identical tartrate to copper ratios, for different copper levels, shows that the readings are virtually the same. One can conclude, then, that the effect of tartrate depends not on its actual concentration, but upon the relative amounts of tartrate and copper.

It will already be clear that the behavior of a biuret reagent is affected by its content of copper, alkali, and tartrate. An attempt is made to illustrate the interrelationships of all three of these factors in Fig. 6. Here a comparison is made of the color developed upon addition of serum to reagents containing two levels of copper, each with 3 and 10 times the

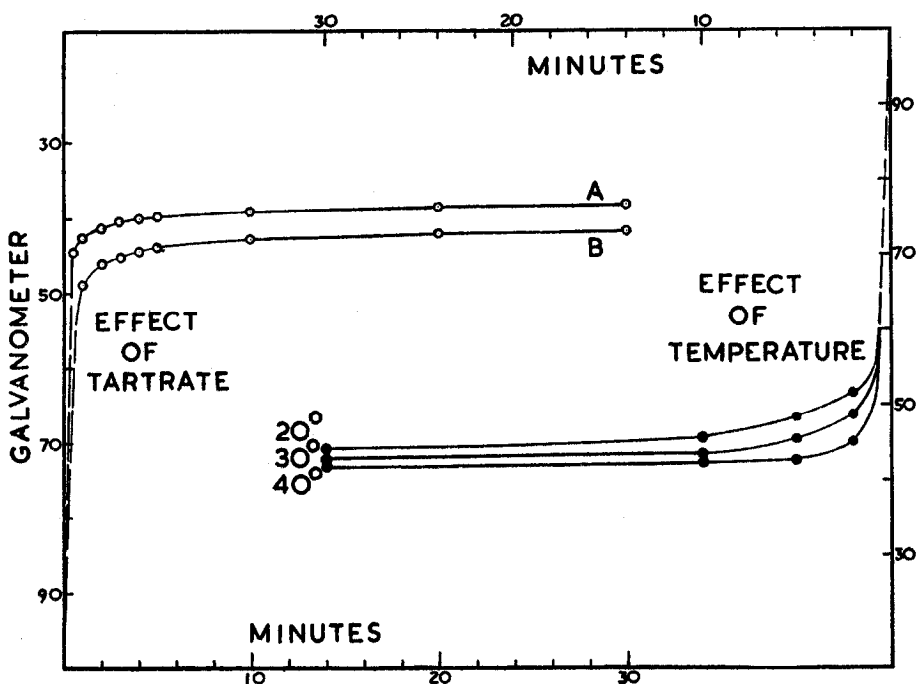


FIG. 4. Rate and degree of color development in biuret reaction mixtures. O, Curve A, without tartrate, Curve B, with 0.48 per cent sodium potassium tartrate; sodium hydroxide 6 per cent; protein 0.065 per cent. ●, the effect of temperature on a reaction mixture containing 0.12 per cent copper sulfate, 0.48 per cent tartrate, and 2.4 per cent alkali; protein 0.063 per cent.

amount of tartrate, and all four at alkali levels of 0.4, 2.4, and 4.8 per cent. All concentrations shown are those in the final reaction mixture.

From a consideration of Fig. 6 the following statements can be made: (1) In all cases the optical density is greater with the lower tartrate to copper ratio. (2) At the 0.4 per cent alkali level, 0.12 per cent copper sulfate is better than 0.32 per cent for either tartrate ratio. (3) With 2.0 to 2.5 per cent sodium hydroxide, there is no difference between copper levels of 0.12 and 0.32 per cent at a 3:1 tartrate ratio, but 0.12 per cent copper is still somewhat better for a 10:1 ratio. (4) When the alkali concentration is about 5 per cent, the reagent with 0.32 per cent copper

is somewhat better for a 3:1 tartrate ratio, but 0.12 and 0.32 per cent copper sulfate are virtually identical in reagents containing 10 times these amounts of tartrate.

Another important property of biuret reagents stabilized with tartrate is the absence of any serious tendency to develop turbidity in the reaction mixture following the addition of clear serum or plasma. Thus one of the main difficulties of Kingsley's reagent, and a concern with Mehl's procedure, is avoided. Clouding will occur in situations in which the protein concentration is excessively high in relation to the copper content

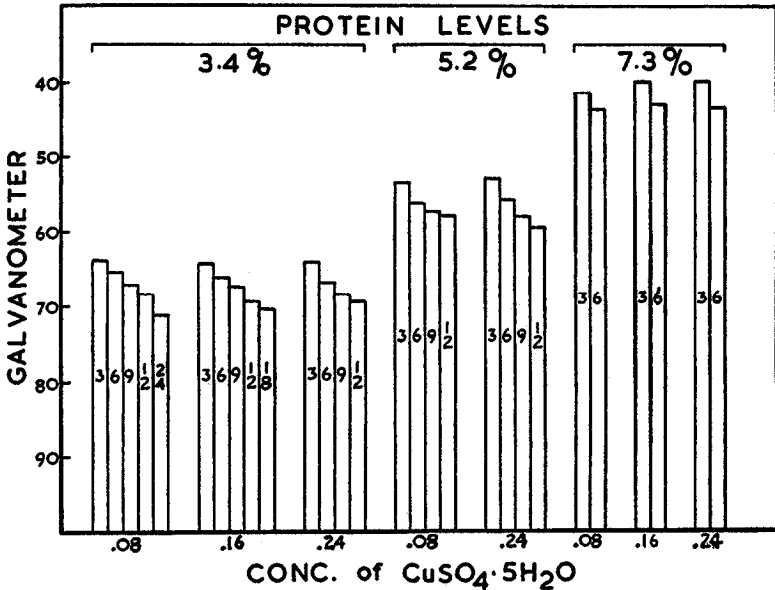


FIG. 5. Effect of the tartrate to copper sulfate ratio, indicated within the columns, on the degree of color development. Serum protein levels and final copper concentrations as shown. Sodium hydroxide 2.4 per cent.

of the reagent. We feel safe, however, in asserting that turbidity will not arise at final 1 in 100 dilutions of sera containing up to 15 per cent protein when the copper sulfate level is 0.12 per cent and the tartrate ratio 4:1. Tartrate is without effect on the precipitate produced by 3 per cent alkali after 12 to 24 hours.

*Use of Potassium Iodide*—Shaffer and Somogyi observed that, in tartrate-containing copper reagents for the determination of reducing sugar, potassium iodide served to prevent "autoreduction" and separation of cuprous oxide. At room temperature, deterioration of the reagent was prevented by 1 gm. of potassium iodide per liter. For maximal stability

during periods of heating, however, it was recommended that 5 gm. per liter be added. Weichselbaum has incorporated 5 gm. of potassium iodide per liter in his reagents.

We have studied the effect of omitting potassium iodide entirely and, with more than 100 different preparations of the reagent, have encountered cuprous oxide precipitation in only one instance. This reduction, and

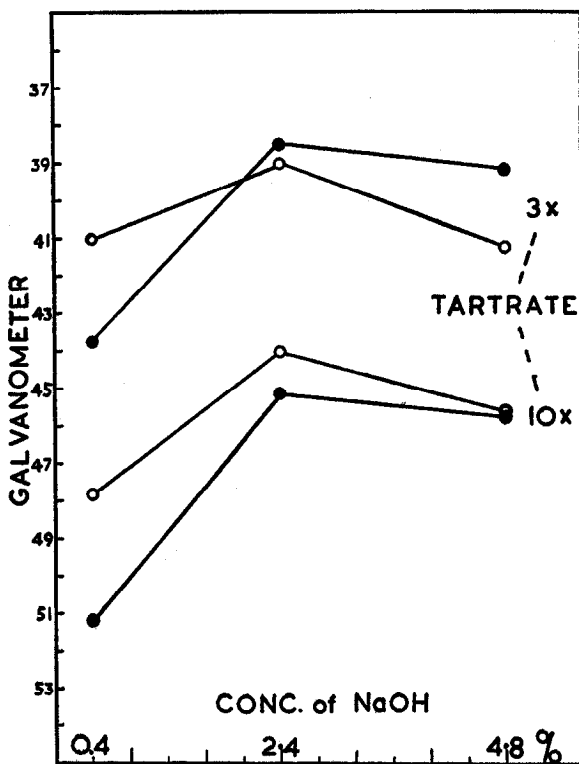


FIG. 6. The interrelationships of copper, tartrate, and alkali concentrations in the biuret reagent. O, 0.12 per cent copper sulfate, ●, 0.32 per cent copper sulfate.

indeed the so called autoreduction, we feel bound to regard as due to some contamination, or impurities in the chemicals used. It is well known that potassium iodide favors the reoxidation of reduced copper and may therefore mask the presence of such impurities. It would seem that potassium iodide can, with reasonable safety, be omitted from the reagent. If, under conditions of preparation or use, any tendency to reduction should be noted, 1 gm. of potassium iodide can be included in each liter of reagent. The presence of this amount of iodide has no detectable effect upon the rate, degree, or quality of biuret color production.

*Choice of Biuret Reagent*—It is plain that there are many biuret reagents, differing slightly in composition, which will work with comparable accuracy and satisfaction if they meet the conditions defined above. The data presented have led us to prefer a reagent containing 0.15 per cent copper sulfate, 0.6 per cent sodium potassium tartrate, and 3.0 per cent sodium hydroxide (with 0.1 per cent potassium iodide optional). When this reagent is mixed in a proportion of 8:2 with 1 in 20 dilutions of serum, the final reaction mixture contains four-fifths of the above concentrations. Such a reagent works exceedingly well in the Evelyn photoelectric and certain other colorimeters. With instruments that are somewhat less sensitive, we recommend that 3 ml. of diluted protein solution be used with 7 ml. of a reagent containing 0.25 per cent copper sulfate, 1.0 per cent sodium potassium tartrate, and 3.5 per cent sodium hydroxide. If readings are made in a single cuvette requiring 10 ml. of solution, the volume of reagent can be increased to allow 2 to 3 ml. of fluid for rinsing.

#### *Determination of Serum Total Protein, Albumin, and Globulins*

An adaptation of the biuret method to the estimation of protein fractions, separated by Kingsley's (6) modification of Howe's procedure (but at a 1 in 20 dilution), is described in detail. The same general technique can be applied to protein fractionations by the sulfite method of Campbell and Hanna (15), the alcohol method of Pillemer and Hutchinson (16), or the modified sulfate method of Majoor (17).

#### *Reagents*—

*Biuret reagent.* Weigh 1.50 gm. of cupric sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) and 6.0 gm. of sodium potassium tartrate ( $\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ ); transfer to a dry 1 liter volumetric flask, and dissolve in about 500 ml. of water. Add with constant swirling 300 ml. of 10 per cent sodium hydroxide (prepared from stock, carbonate-free, 65 to 75 per cent sodium hydroxide solution). Make to volume with water, mix, and store in a paraffin-lined bottle. This reagent should keep indefinitely but must be discarded if, as a result of contamination or faulty preparation, it shows signs of depositing any black or reddish precipitate.

*Globulin precipitants.* (a) Sodium sulfate, 22.6 per cent; (b) ethyl ether. Sodium chloride, 0.9 per cent.

#### *Procedure*

Select three colorimeter cuvettes (or test-tubes) and mark them Cuvettes B (blank), T (total protein), and A (albumin).

Into Cuvette B pipette 2.0 ml. of 22.6 per cent sodium sulfate solution. This "blank" will serve for all the protein analyses being carried on at any one time.

Into a centrifuge tube measure, with an Ostwald pipette, 0.5 ml. of serum and add 9.5 ml. of 22.6 per cent sodium sulfate. Stopper the tube and mix thoroughly by inversion (not by shaking). At once transfer 2.0 ml. of the mixture to Cuvette T.

To the remaining serum-sulfate mixture add 3 ml. of ether, mix for 30 seconds, cap, and centrifuge. Slant the tube and transfer 2.0 ml. of the aqueous phase to Cuvette A.

Now into each of the three cuvettes pipette 8.0 ml. of biuret reagent and mix thoroughly by swirling. Allow these to stand for 30 minutes at room temperature (20–25°).

Using a photoelectric colorimeter, or spectrophotometer, transmitting maximally at 540  $m\mu$ , adjust it to 100 per cent transmission with the "blank" Cuvette B in position. Replace Cuvette B with Cuvettes T and A in turn and record the percentage transmission (or optical density) of each.

Obtain the concentration of total protein and of albumin in the serum by reference to the calibration curve; the total protein minus the albumin gives the globulin concentration.

*Calibration Curve*—Pipette 5.0 ml. of clear, normal serum into a stoppered, graduated cylinder, dilute to 50 ml. with 0.9 per cent sodium chloride, and mix.

Prepare in duplicate a series of nine cuvettes (or test-tubes) and into them pipette successively 2.0, 1.85, 1.70, 1.55, 1.40, 1.20, 1.00, 0.80, and 0.60 ml. of 0.9 per cent saline. Then, in the same order, add 0, 0.15, 0.30, 0.45, 0.60, 0.80, 1.00, 1.20, and 1.40 ml. of the diluted serum.<sup>3</sup>

Now pipette into each cuvette 8.0 ml. of biuret reagent, mix, and read after 30 minutes as above.

Kjeldahl nitrogen determinations can be carried out on the remaining diluted serum. The total nitrogen content of 100 ml. of serum, corrected for non-protein nitrogen from a separate determination on the original serum, gives protein nitrogen which, multiplied by 6.25, is taken as the standard protein concentration.

Plot the transmission or density values observed against 0.15, 0.30, 0.45, 0.60, 0.80, 1.0, 1.2, and 1.4 times the protein concentration of the standard serum.

### Results

The calibration curves we have obtained using the reagent and the method described above obey satisfactorily the laws of Lambert and Beer.

<sup>3</sup> For work with 3.0 ml. samples use 0, 0.24, 0.45, 0.66, 0.9, 1.2, 1.5, 1.8, and 2.1 ml. of diluted serum, made to 3.0 ml. with saline. Plot the transmission values against 0, 0.16, 0.3, 0.44, 0.6, 0.8, 1.0, 1.2, and 1.4 times the protein concentration of the standard serum.

A slight loss of blue color must occur in the reaction because a proportion of the copper enters the protein "biuret" complex, but the effect on the extinction coefficient is scarcely apparent even at serum protein concentrations around 10 per cent. In Fig. 7 a typical calibration curve is shown, plotted on semilogarithmic paper. With the Evelyn colorimeter our  $K$  value ( $(2 - \log G)/\text{concentration}$ ) has averaged about 580 with a range in any one curve of  $\pm 10$ .

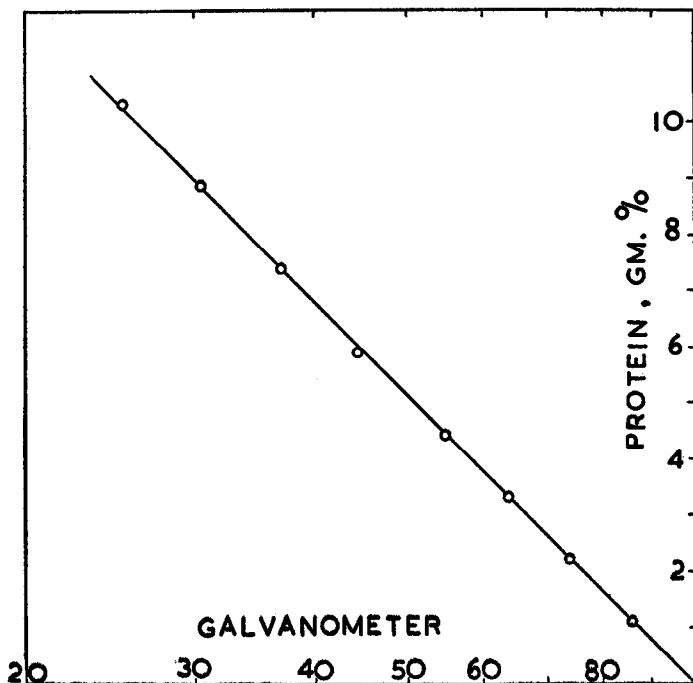


FIG. 7. Calibration curve for protein estimations with the biuret reagent, plotted on semilogarithmic graph paper.

Total protein and albumin analyses made with the modified biuret reagent have been compared with values calculated from Kjeldahl nitrogen determinations on the same sera; the results are shown in Table II. It can be seen that the biuret and Kjeldahl results can be expected to agree within about 0.1 gm. per cent of protein. The observed differences are statistically insignificant.

#### DISCUSSION

In our experience, the use of tartrate-stabilized biuret reagents offers a highly satisfactory method for estimating the proteins of serum or plasma.

The values obtained depend ultimately upon a Kjeldahl nitrogen determination, hence upon an ability to perform such an analysis accurately, and with the assumption of the factor 6.25. At present there appears to be no practical method of standardization superior to nitrogen analysis.

TABLE II

*Results of Comparison of Total Protein and Albumin Analyses by Biuret and Kjeldahl Methods*

The values are measured in gm. per cent.

Serum		Diagnosis	Total protein		Albumin	
			Kjeldahl	Biuret	Kjeldahl	Biuret
Human	A. G.	Normal	7.49	7.35		
	C. B.	"	7.01	7.05		
	B. B.	"	7.04	7.05		
	J. D.	"	6.70	6.60		
	Pooled	"	7.45	7.35		
	H. G.	"	7.62	7.80		
	F.	Pyelonephritis	4.50	4.50	1.40	1.40
	R.	Pneumonia	7.12	6.95	3.79	3.81
	G.	Rheumatoid arthritis	6.80	6.70		
	C.	Cirrhosis	6.97	7.05		
	L.	Obstructive jaundice (serum bilirubin, 25 mg. %)	5.81	5.82		
	M.	Obstructive jaundice	5.91	6.07		
E.	Lipoid nephrosis	3.85	3.65			
T.	Lupus erythematosus			3.25	3.20	
Dog No.	11	Normal	5.67	5.70		
	15	"	5.84	5.85		
	17	"	6.60	6.60		
	10	CCl <sub>4</sub> liver injury	6.08	5.95	3.35	3.24
	12	" " "	7.20	7.15		
	16	" " "	6.45	6.40		
	17	" " "			2.72	2.88
	48	Uremia (non-protein N = 200 mg. %)	5.06	5.15		

The procedure described for total protein determinations involves re-dissolving the globulins precipitated by sodium sulfate or other agents. No difficulty has been experienced with this technique, nor has the presence of sulfate any effect upon the rate or degree of color development. Sodium sulfite and methanol (at a final concentration of 2.1 per cent) may cause a very slight increase in color intensity, but for practical purposes the effect is negligible.

It is pertinent to comment briefly on a few special points.

*Interfering Factors*—There are practically no substances other than protein in biological fluids which give the biuret reaction, certainly none that cause significant interference. The pigment, bilirubin, absorbs light very weakly at 540  $m\mu$ . We confirm Kingsley's (6) observation that serum levels as high as 25 mg. per cent introduce (at the dilution employed) an error not greater than 0.1 gm. per cent protein. Although ammonium ion is a disturbing factor in the biuret reaction, the amount present when a mixture of ammonium and potassium oxalates is used as anticoagulant will not cause any significant error. With the method described, ammonium sulfate cannot be used as a globulin precipitant, but its interference can be minimized (a) by analysis of the separated precipitate or (b) by using a reagent containing 8 to 10 per cent alkali.

Careful separation of the serum or plasma should avoid the disturbing effect of hemolysis, and, if the patient is in the fasting state, the specimen will almost always be clear. There are certain diseases and situations, however, which give rise to lipemic sera and it is then necessary to vary the technique somewhat if the results are to have any significance. Such sera yield a somewhat cloudy reaction mixture and clearing must be effected before readings can be made. For this purpose we have used Kingsley's (6) method of adding 3 ml. of ether. The phases are mixed by flicking the tube for a definite period, say 10 or 20 seconds; the mixture is then centrifuged in a capped tube and the aqueous phase transferred to a new cuvette for reading at 30 minutes. This technique introduces a dilution error which in our hands has amounted to about 3 per cent. It is necessary for each worker to establish this correction, not only by measuring the change in volume, but by noting the optical density of a clear reaction mixture before and after treatment with ether. The use of petroleum ether (ligroin, b.p. 70–90°) will avoid the dilution error, though occasionally the two phases may not separate quite so readily.

*Age of Serum*—Wokes and Still (18) have reported that the intensity of the biuret color reaction increases rather strikingly as plasma ages. Our own experience is not in accord with their findings. If serum, or oxalated plasma, has been removed within a reasonable time ( $\frac{1}{2}$  to 2 hours) and stored under conditions in which it remains clear, no significant change has been observed in the protein concentration determined by the biuret reaction at  $\frac{1}{2}$ , 2, 4, 24, or 168 hours.

*Biuret Reaction with Different Proteins*—Although the exact nature of the "biuret" complex is still unknown, it is apparent that the accurate determination of serum protein fractions must presume a constant, average number of groupings which combine to give the reaction. Evidence supports the view that in this respect the biuret reaction is a more reliable quantitative method for protein estimation than one depending on the

content of some particular amino acid. Autenrieth (1) and Robinson and Hogden (5) regarded the color development by serum albumin and globulins as virtually identical. Fine (3), Kingsley (12), and Wokes and Still (18) have reported apparent differences in behavior of these two fractions in the biuret reaction.

We have determined the  $K$  values  $((2 - \log G)/C)$ , which are comparable to extinction coefficients, for the proteins listed in Table III. The protein concentration ( $C$ ) in each case has been calculated from a Kjeldahl nitrogen determination, corrected for non-protein nitrogen, and with the factor 6.25. The  $K$  values differ from the 580 previously mentioned because the reagent used for this experiment was of slightly different composition. It will be noted that, among the proteins tested, all the albumins and

TABLE III  
*K* Values for Different Proteins, Comparable to Extinction Coefficients

Nature of protein	$K = \frac{2 - \log G}{C}$
Human serum albumin	520
“ “ globulins	514
“ “abnormal” serum globulin (pptd. with 13.5% Na <sub>2</sub> SO <sub>3</sub> )	515
“ serum (pooled)	520
Dog “ “	517
Egg albumin	513
Casein	458
Zein	479
Gelatin (animal)	386

globulins show essentially identical values. The results with casein and zein differ to some extent, while gelatin shows a rather striking difference, one which was apparent visibly as a bluer tint.

The authors gratefully acknowledge the valued suggestions made by Dr. Andrew Hunter during the preparation of this report.

#### SUMMARY

Among the methods of preparing stable, “one piece” biuret reagents, the use of sodium potassium tartrate appears most promising.

The tartrate-stabilized reagent devised by Weichselbaum has been investigated in some detail and has been modified to contain less copper, more alkali, and less or no potassium iodide.

A simple procedure for the determination of serum total protein, albumin, and globulins is described.

The biuret reaction is approved as a simple, rapid, yet highly satisfactory and accurate method for the determination of the protein fractions in serum or plasma.

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