THE DETERMINATION OF SERUM PROTEIN CONCENTRATION WITH A GRADIENT TUBE

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The specific gravity of serum has been shown to be a reliable index of serum protein concentration (1, 2). The methods which appear to be best suited for the rapid determination of serum specific gravity are the falling drop method of Barbour and Hamilton (3), the copper sulfate procedure of Phillips *et al.* (4), and the gradient tube method of Linderstrøm-Lang. Although the use of the gradient tube for very precise specific gravity measurements has been carefully described (5), there have been but brief references to the adaptation of this method to the rapid measurement of serum or plasma specific gravity (6–8).

The simplicity, convenience, speed, and accuracy of the gradient tube method recommend it not only for routine hospital use and experimental purposes, but the method has been found to be almost ideally suited for large scale population studies of protein nutrition. For such survey work the fact that only 2 or 3 c.mm. of serum are required is particularly advantageous. It appears, therefore, desirable to describe the method more fully, and to give the results of 240 comparisons of serum protein values determined by the gradient tube with the protein concentrations calculated from the total nitrogen (Kjeldahl).

Details of collecting serum from the finger and comparisons between serum protein values obtained from finger, ear, and venous blood will be given, and the effects of storage, etc., will be discussed.

Method

Principle—By the use of mixtures of different proportions of a heavy and a light organic liquid, a linear density gradient is established in a graduated cylinder. Droplets of serum, 2 to 4 c.mm. in size, are allowed to fall into this gradient, and they come to rest upon reaching a point having a density exactly equal to their own. The density, and hence the protein concentration, are calculated graphically by interpolation from the position of droplets of salt solutions of known density. To prevent disturbances of the gradient tube by convection currents, the cylinder is installed in a jar of water.

Materials—

1. *Gradient tube and water jacket* (Fig. 1), a special 500 ml. graduated cylinder (*A*) fitted inside a larger cylinder (*B*). (This may be obtained from Eimer and Amend, New York, or may be easily constructed in the laboratory from readily available material; see Lowry and Hastings (7).)

2. *Gradient Solution A*, 100 ml. of bromobenzene (sp. gr. 1.49), technical grade, plus 150 ml. of kerosene (sp. gr. 0.80); white kerosene is preferable, although special purity is not required. If the specific gravity is not 1.07 ± 0.005 (urinometer), adjust to 1.07 with a little bromobenzene or kerosene.

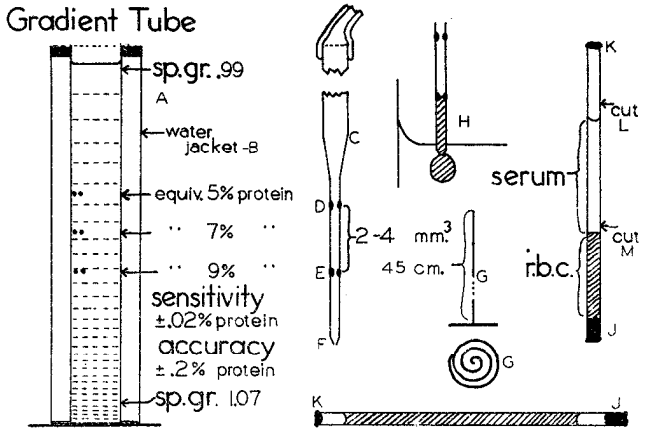

FIG. 1. Gradient tube for determination of serum protein concentration. *A*, graduated cylinder; *B*, water jacket; *C*, constriction pipette; *D*, *E*, and *F*, constrictions; *G*, copper wire stirrer; *H*, insertion of droplets; *J* and *K*, picene seals on blood-collecting tubes.

3. *Gradient Solution B*, 70 ml. of bromobenzene plus 180 ml. of kerosene. Adjust, if necessary, to a specific gravity of 0.99 ± 0.005.

4. *Jacket solution*, 0.2 per cent copper sulfate in 0.1 per cent sulfuric acid to fill the space between the two cylinders above. (The copper sulfate prevents molds and absorbs radiant heat.)

5. *Constriction pipettes* (Fig. 1, *C*), similar to those described by Linderstrøm-Lang (5) but adapted to deliver 2 droplets instead of 1. The constrictions differ in size, the upper one, *D*, being a little larger than the middle one, *E*, which is in turn larger than the bottom constriction, *F*. (These are obtainable from Eimer and Amend, New York, or may be constructed in the laboratory with a little practice, by the use of an injection needle (size 22 to 25) as a micro burner to make the constrictions.)

6. *Stirrer*, a wire spiral made from heavy copper wire, 12 to 16 gage (Fig. 1, *G*) (suggested by Dr. A. M. Butler).

7. *Blood-collecting tubes*, melting point capillaries, *H*, of 1.5 to 2.0 mm. outer diameter, 7 to 10 cm. long, open at both ends, for collecting blood (*e.g.* No. 34500 of the Kimble Glass Company, Vineland, New Jersey).

8. *Picene cement* for sealing the capillaries.[1]

9. *Fine sea sand* for removing droplets from the gradient tube.

10. *Specific gravity standards.*[2] 250 gm. of C.P. K_2SO_4 are dried overnight at 100–110°. 17.64, 23.08, 28.53, 34.04, 39.58, and 45.10 gm. amounts are weighed out (tolerance, 0.05 gm.) and each sample is diluted to 1 liter to give six standard solutions of specific gravity, 1.0141, 1.0184, 1.0227, 1.0270, 1.0313, and 1.0356, which are equivalent to serum protein concentrations of 2.5, 4.0, 5.5, 7.0, 8.5, and 10.0 per cent, respectively. These specific gravities are calculated *relative to water at the same temperature.* These standards will keep indefinitely if protected from mold and evaporation by storing in rubber-stoppered bottles in the ice box. For working standards, 5 or 10 ml. rubber-stoppered bottles or tubes are filled with these solutions. Every week or two the small vessels should be emptied and refilled from the large samples in the refrigerator.

Preparation of Gradient Tube—The graduated cylinder is filled to the middle graduation with *Solution A*, and on top of this *Solution B* is carefully layered to the upper graduation. This may be accomplished by filtering *Solution B* into the cylinder, arranging the funnel so that the solution runs down the cylinder wall. The space between the two cylinders is filled with the dilute acid copper sulfate.

The density gradient is now established by partially mixing the two layers with the copper spiral, *G*, in the following manner. Strokes of uniform velocity are made with the spiral between positions 3 or 4 cm. above and below the middle graduation until the schlieren effects (disturbances in transmitted light due to differences in refraction) are of nearly the same magnitude throughout the interval. The strokes are then lengthened by steps of 6 or 8 cm. until they reach the full length of the column. At each step, mixing is continued until the schlieren effects are of the same magnitude throughout the interval. It will perhaps be surprising to find how much stirring this requires. The usual mistake is to stir too little rather than too much. After 5 or 10 minutes, droplets of each of the standard solutions are put into the cylinder and the positions at which the droplets stop are noted. The total interval between the lightest and the heaviest standard should be one-half to one-fourth of the graduated length. The standard droplets should be nearly equally spaced (±20 per cent). If the droplets are too close together, more full length strokes should be made. If the gradient does not approximate linearity, it may

[1] Pyseal, Eimer and Amend, New York, or Plicene, Central Scientific Company, Boston.

[2] These may be obtained ready made from Eimer and Amend.

be brought into linearity by stirring more when the standards are too close together, though this will seldom be necessary. If the droplets are too widely spaced, there has been too much stirring. In this event the gradient should be reestablished as follows: The bromobenzene and kerosene are siphoned or poured out, completely mixed, and divided into two equal parts. Each part will now have a specific gravity of about 1.03. To one part are added 95 ml. of bromobenzene per liter to bring it to 1.07 specific gravity. To the other portion are added 210 ml. of kerosene per liter to bring it to a specific gravity of 0.99. With these as the starting solutions, the cylinder is filled as before and the gradient is reestablished as described above.

After the cylinder has stood for half an hour, the gradient is ready for use. It should be set on a level table out of direct sunlight and away from other sources of heat. A light background will facilitate reading. The gradient will improve with time, becoming more nearly linear. Eventually the intervals between standards will become greater and finally, after 6 months to a year, depending on use and protection from evaporation, the heaviest standard will fall through to the bottom. When this occurs, the gradient should be reestablished as described above. In warm weather the standards will all lie lower than in cold weather, since the temperature coefficient of water is much less than that of the organic solvents. If the gradient tube is not kept tightly stoppered when not in use, the more volatile bromobenzene will evaporate somewhat.

Use of Gradient Tube—The constriction pipette (Fig. 1) is rinsed two or three times and filled to the upper constriction with the heaviest standard, a rubber tube being used as with a blood-counting pipette. The pipette tip is gently wiped on a slightly moistened piece of filter paper or towel and introduced into the gradient tube on the extreme left side. With the tip just below the liquid surface and about 5 mm. from the wall, the solution is blown down to the middle constriction where surface tension stops it. By raising the tip of the pipette through the surface, the droplet pulls away and falls. The tip is put back into the liquid and the second droplet is delivered a few mm. behind the first. Care is taken not to blow air after the second droplet, as this would break it into a fine spray.

The rest of the standards are put directly on top of the first in the order of decreasing specific gravity. The pipette is rinsed several times with each new standard before use. For nearly normal sera the 5.5, 7.0, and 8.5 per cent protein standards will suffice. The positions of the standard droplets are read 4 minutes or more after the last standard has been introduced, by which time all will have reached equilibrium. It is more convenient and accurate to read the position of the lower edge of each droplet than to try to estimate the height of its center.

Serum is delivered in the same manner as the standards. Separate pipettes should be used for the standards and for serum, since serum leaves a film on the pipette which makes it difficult to pipette salt solutions. If there is insufficient serum available to rinse the pipette, it may be rinsed with water and dried with acetone before use. There is ordinarily little or no error if different sera are pipetted one after another without rinsing. If a pipette that has been used for serum remains idle for more than a moment, it should be rinsed with water, since otherwise the fine tip may dry shut. A dried tip can usually be reopened with concentrated nitric acid.

The serum droplets are delivered into the gradient tube a few mm. to the right of the standards. A duplicate droplet is unnecessary if there is a shortage of serum, but its presence increases confidence in the result and adds scarcely at all to the time consumed. Each serum is introduced in a separate "lane" a few mm. to the right of the previous one. Vertical marks at the top of the cylinder aid in placing the droplets in separate lanes. As many as ten or twenty serum samples may be inserted if care is taken to place them close to each other. If desired, a second row of droplets may be inserted 2 or 3 cm. behind the first. Each pair of droplets is read 4 $\pm$ 0.5 minutes after delivery. In the interval, the next three or four samples may be put in. With experience, no difficulty is encountered in keeping track of the time and order of the droplets. The positions of the standards are plotted on graph paper against their equivalent serum protein concentration, and from the resultant curve the unknown sera are evaluated. It is unnecessary to consider the temperature of the gradient tube at all, since both serum and standards have essentially the same temperature coefficient.

Removal of Droplets—After the column becomes full of droplets a little of the sea sand is sprinkled into the top of the gradient tube. The fine particles adhere to the droplets, carrying them to the bottom, and the gradient may be used again immediately.

Turbidity—If the gradient tube should ever become turbid (due to moisture), it may be clarified by grinding a little $CaCl_2$ with a few ml. of the top gradient mixture and quickly pouring the resultant suspension into the column. It will be necessary to wait some hours after this before using the gradient again, in order to give time for the finer particles of $CaCl_2$ to settle out.

Collection of Small Serum Samples—Since only a few c.mm. are required for serum protein determination with the gradient tube, it is possible to use the following simple method of serum collection. Capillary tubes, 1.5 to 2.0 mm. in outer diameter and 7 to 10 cm. long, are provided. Blood is taken from the finger or ear lobe, with care to wipe off the first droplet of blood and all traces of alcohol before taking the sample. Squeezing the

finger is permissible (see below). The capillary tube is about three-quarters filled by capillarity.[3] The tube is tipped until the blood runs to the middle, care being taken to keep one end of the capillary dry. The ends are sealed as follows: The stick of picene is softened in a flame (match, alcohol lamp, etc.) and applied to the *dry end*, *J*, of the capillary which has also been warmed in the flame. For the tight seal required at this end, the glass must be warm enough for the picene to "wet" it. The other end of the capillary, *K*, is capped with a little picene without warming the tube, which might otherwise produce hemolysis. The capillary is centrifuged 5 to 10 minutes at 3000 R.P.M. with the tightly sealed end down. A great number of samples may be centrifuged together by wrapping each tube in

TABLE I

Blood Samples Taken by Venipuncture and Capillary Puncture from One Individual within Short Space of Time

The values are given in gm. per cent. Each value represents a different capillary tube. The figures for finger and ear are given in the order in which they were taken.

Source of blood	Remarks	Serum protein	Source of blood	Remarks	Serum protein
Vein	Macro sample	6.76	Finger Puncture 2	Free flow	6.80
"	Centrifuged in capillary tubes	6.77		" "	6.63
		6.83		" "	6.65
		6.78		" "	6.66
Finger Puncture 1	Squeezing	6.72	Finger Puncture 3	Hard squeezing	6.76
	Slight squeezing	6.69		" "	6.76
	Free flow	6.65		" "	6.74
	" "	6.67	Ear	Free flow	6.72
	Squeezing	6.67		" "	6.76
				" "	6.76
				" "	6.76

a small piece of paper bearing identification. The protein should be measured within 8 hours if kept at room temperature, or within 48 hours if kept at 4–6°. When ready to make the determination, the top of the capillary above the serum is removed after scratching at *L* (Fig. 1) with a diamond point. A second scratch, *M*, is made just above the red cells and the serum segment is removed. The serum is drawn into the constriction pipette and transferred to the gradient tube as described above.

Venous Versus Capillary Blood—Table I demonstrates the correlation of blood samples taken from one individual by vein, finger, and ear. The

[3] This will furnish 4 or 5 times the amount of serum required for the protein determination; hence, if no other analyses are contemplated, a much smaller amount of blood may be taken.

venous samples were obtained from a large blood specimen, part of which was centrifuged as a large sample and part of which was transferred to capillary tubes for centrifugation. It would appear that centrifuging in capillary tubes does not change the specific gravity and that capillary blood and venous blood yield identical serum protein values within 0.1 gm. per cent. Furthermore, squeezing is without influence on the result obtained. This suggests that the well known influence of squeezing on hematocrit values is due to dilution with serum rather than to dilution with tissue extracellular fluid of lower protein concentration. Except perhaps in the case of Finger Puncture 2, the first sample agrees with the rest. Nevertheless, it would seem desirable in general to discard the first droplet as is done in taking blood for enumeration of red cells.

Effect of Storage—As with larger samples, the storage of whole blood in capillary tubes eventually changes the serum protein concentration (Table II). As might be anticipated, the changes occur more rapidly at room

TABLE II

Effect of Storage of Whole Blood in Capillary Tubes on Serum Protein Values

Temperature	Time stored				
	0 hr.	2 hrs.	6.5 hrs.	25 hrs.	48 hrs.
	Protein concentration, gm. per cent				
°C.					
30	6.83	6.93	6.90	7.30	7.52
4	6.83	6.89	6.88	6.87	6.91

temperature than at 4°. At the lower temperature there was little change in 48 hours. At 30° there was no significant change in 6 hours, but a definite increase in serum density occurred within 25 hours. This change is presumably the consequence of the swelling of red cells with attendant concentration of the serum, resulting from glycolysis.

Correlation with Kjeldahl Determination—In 240 individuals without obvious liver damage[4] there was found to be a standard deviation of 0.24 gm. per cent between serum protein values obtained with the gradient tube and those calculated from the total nitrogen (Kjeldahl). This correlation was obtained with the formula, per cent protein = 348 (sp. gr. 1.0069). This formula was employed since it gave slightly better correlation with the Kjeldahl values than either the formula of Moore and

[4] Cases with liver damage have been omitted because in many instances the gradient tube gave distinctly lower values (average 0.2 per cent) than were obtained by Kjeldahl determination. One of the authors (T. H. H.) will elaborate on this finding elsewhere.

Van Slyke (1) (protein = 343 (sp. gr. 1.007)), or that of Weech, Reeves, and Goettsch (2) (protein = 347.9 (sp. gr. 1.00726)). These latter formulae give serum protein values approximately 0.1 gm. per cent lower than the formula we have used.

Table III shows the correlation of serum protein concentrations obtained with the gradient tube with the values calculated from the total nitrogen. These 240 serum samples were obtained from patients with a variety of diseases. The protein concentrations varied from 3.5 to 13.5 gm. per cent. Many sera with abnormal albumin to globulin ratios are represented. It will be seen that only 5 per cent of the values obtained by the Kjeldahl procedure and the gradient tube deviate by more than ±0.4 gm. per cent. Looney (9) failed to obtain a high degree of correlation between serum specific gravity and serum protein concentration, but unfortunately his serum protein values are based on a turbidimetric procedure.

TABLE III

Correlation of Serum Protein Values Determined with Gradient Tube with Those Determined by Kjeldahl Analysis in 240 Normal and Pathological Sera

Deviation between methods, *gm.* %	±0.0	±0.1	±0.2	±0.3	±0.4	±0.5	±0.6	±0.7
% of determinations with given deviation	16	37	20	14	8	2	2	1
Cumulative % of determinations deviating more than this given deviation	84	47	27	13	5	3	1	0

DISCUSSION

From the foregoing it should be possible to assess the advantages and disadvantages of the gradient tube for the determination of serum protein. The advantages appear to lie in the smallness of the sample required, the speed of analysis, the freedom from influence of temperature or size of drop, and the lack of necessity for accurate timing. The $CuSO_4$ method (4) requires several hundred c.mm.,[5] the falling drop method 10 c.mm.,

[5] We have observed a discrepancy between the specific gravity measured by the gradient tube and by the copper sulfate method. For ten serum samples analyzed both ways the copper sulfate method gave specific gravity values *lighter* by an average of 0.0016 (standard deviation 0.0003). In comparison with the pycnometer the gradient tube appears to yield specific gravity values *too heavy* by about 0.0005, whereas in our hands the copper sulfate method gives values which appear to be *too light* by about 0.0010. Recently (10) a new formula has been introduced for use with the copper sulfate method which brings results by the two methods more nearly into agreement (per cent protein = 360 (sp. gr. 1.007) instead of per cent protein = 343 (sp. gr. 1.007)).

whereas for the gradient tube only 2 to 3 c.mm. are required. Indeed, if desirable, as little as 0.5 c.mm. in a smaller pipette will suffice.

Approximately one determination in duplicate per minute may be made with the gradient tube. This is somewhat faster than with any other specific gravity method. The freedom from dependence upon temperature, size of drop, and accurate timing is shared by both the gradient tube and $CuSO_4$ methods in contrast with the falling drop procedure. Perhaps these have been the chief disadvantages of the falling drop method.

The gradient tube shares in common with all other serum protein methods that are based on measurements of specific gravity the disadvantage that serum constituents other than protein influence serum density. Although this point will be discussed more fully in a subsequent paper, it may be noted that in order to influence the apparent serum protein concentration by as much as 0.1 gm. per cent it would be necessary to double the normal concentration of either the serum lipids, the blood glucose, or the non-protein nitrogen. Cholesterol is almost without effect, since its specific gravity is close to that of serum. With high serum lipid concentrations an error is encountered if the droplets are not read promptly at 4 minutes, since the droplets will fall further with time, presumably due to an absorption of bromobenzene. The data presented in this paper may be taken to indicate that it is rare for any of these factors to affect seriously the results obtained.

The gradient tube method has received extensive trial in the laboratories from which this report originates. To date approximately 5000 protein determinations have been made on sera, from clinical patients (2500), small laboratory animals (1000), and subjects of nutritional surveys (1500). The method has continued to give satisfaction throughout.

Use of Gradient Tube for Determination of Hemoglobin—The specific gravity of whole blood has been shown to be an accurate measure of its hemoglobin content, particularly if the serum specific gravity is also known (Phillips *et al.* (4)). The gradient tube has proved to be very convenient for this purpose and has been especially valuable in field studies when a colorimeter was not available. Only 4 or 5 c.mm. of blood are required for a duplicate determination. In the field, a cylinder (100 or 250 ml.) may be used without a water jacket, to save space. The only changes necessary are in the specific gravity range of the column and in the density standards. The lighter organic mixture is 69.5 volumes per cent kerosene, 30.5 per cent bromobenzene (sp. gr. 1.010); the heavier mixture is 55.0 per cent kerosene, 45.0 per cent bromobenzene (sp. gr. 1.110). The standards contain 57.13, 70.22, 83.49, and 96.86 gm. of K_2SO_4 per liter with a specific gravity of 1.0450, 1.0550, 1.0650, and 1.0750. When needed for very low hemoglobin values, the heaviest serum protein

standard (1.0356) may also be used. The gradient is produced and used exactly as described for serum protein. For simplicity, blood is drawn from the finger into a capillary tube (see above) having a small rubber bulb on one end. This rubber bulb is the type used for vaccination purposes and is perforated at both ends. The second perforation allows blood to enter the tube by capillarity, but on closure of this hole with the finger and squeezing the bulb, 2 droplets of blood may be delivered directly into the gradient tube. That the 2 droplets will seldom be of the same size is immaterial. The blood must, of course, flow freely from the finger with little or no squeezing. Calculation of the hemoglobin concentration is made from the nomogram of Phillips *et al.* (4).

SUMMARY

1. A density gradient tube suitable for the rapid determination of the specific gravity of 2 to 3 c.mm. of serum is described and directions given for its use.

2. The collection of a small amount of serum from the finger or ear for use in the gradient tube is described.

3. Comparison is made between serum protein concentration in serum from the vein, finger, and ear, and the influence of storage on the results obtained is shown.

4. In 240 individuals without liver damage serum protein values obtained with the gradient tube are correlated with those calculated from total nitrogen determinations. In individuals without liver damage, a standard deviation of 0.24 gm. per cent has been found between results by the two methods.

5. The use of the gradient tube for measuring blood hemoglobin concentration is described.

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