THE DETERMINATION OF ASCORBIC ACID IN SMALL AMOUNTS OF BLOOD SERUM

By OLIVER H. LOWRY, JEANNE A. LOPEZ, and OTTO A. BESSEY

(From the Division of Nutrition and Physiology, The Public Health Research Institute of The City of New York, Inc., New York)

(Received for publication, July 19, 1945)

The measurement of the ascorbic acid level in serum has attained considerable importance as an index of ascorbic acid nutrition. Existing ascorbic acid methods require at least 0.1 ml. of blood serum or 0.2 ml. of blood (1, 2), an amount which can just be obtained from the finger. If, as is usually the case in making nutritional surveys, it is desired to measure a number of other blood constituents, resort must be had to venipuncture to obtain sufficient blood.

The advantage of avoiding venipuncture in dealing with large groups, especially when children are concerned, has led to an exploration of the possibility of determining ascorbic acid on much less than 0.1 ml. of serum.

Three different reagents have been used successfully for measuring ascorbic acid in blood serum: dichlorophenol indophenol (Mindlin and Butler (1)), methylene blue (Butler, Cushman, and MacLachlan (2)), and dinitrophenylhydrazine (Roe and Kuether (3)). The first two reagents measure ascorbic acid directly, whereas dinitrophenylhydrazine measures ascorbic acid only after oxidation to dehydroascorbic acid. All three reagents have been investigated for their adaptability to small scale analyses.

Of the three, the methylene blue reagent is by far the most sensitive, but its use requires an irradiation step which is somewhat awkward in dealing with small volumes. With dichlorophenol indophenol it was found possible to measure the ascorbic acid in 0.01 ml. of serum, but it proved difficult when handling large numbers of determinations to avoid some prior loss of ascorbic acid through oxidation. Therefore, the choice fell upon dinitrophenylhydrazine. Obviously, with this reagent prior oxidation of ascorbic acid to dehydroascorbic acid, if it should occur, would do no harm, since in any event all of the ascorbic acid must finally be converted to dehydroascorbic acid before measurement. It has been found that dehydroascorbic acid (or a derivative which reacts with dinitrophenylhydrazine (4)) is remarkably stable in serum after trichloroacetic acid addition, whether or not the extract is separated from the protein precipitate. This has proved of great advantage in carrying out large numbers of analyses under conditions of a nutritional survey.

It has proved quite simple to adapt the Roe and Kuether dinitrophenylhydrazine method to the measurement of the ascorbic acid in 0.01 ml. of

serum. With the resulting procedure, one person can analyze 50 sera per day. In adapting the procedure to a small scale, it was necessary to make certain simplifications which should prove helpful in large scale work as well.

Method

Reagents and Equipment—

1. 2 per cent dinitrophenylhydrazine, 0.25 per cent thiourea, in 9 N H_2SO_4; centrifuge or filter through sintered glass if a precipitate develops. Add the thiourea only to sufficient reagent for 1 month's use, store in the ice box, and discard the remainder of this portion after 1 month.

2. 65 per cent H_2SO_4 prepared by adding 70 ml. of concentrated H_2SO_4 to 30 ml. of H_2O.

3. 1 per cent suspension of norit in 5 per cent trichloroacetic acid. The norit is washed with acid and dried according to Roe and Kuether (3), and 5 gm. are suspended in 100 ml. of 5 per cent trichloroacetic acid. After settling, the supernatant is decanted and the volume restored with 5 per cent trichloroacetic acid. This is repeated several times to eliminate some of the excessively fine, floating charcoal. Once a week or so the supernatant acid from a small portion is replaced with fresh acid to eliminate the possibility of contamination with heavy metals which, according to Roe and Kuether, may slowly leach out of the norit.[1]

4. Levy-Lang constriction micro pipettes (hand type), 10, 30, and 50 c.mm. (5). In addition, for pipetting the charcoal suspension, a 40 c.mm. pipette with tip and constriction 2 or 3 times wider than normal to avoid plugging with charcoal.

5. A Beckman spectrophotometer fitted with a special diaphragm and cuvettes to permit the use of 0.05 ml. fluid volumes[2] (obtainable from the Pyrocell Manufacturing Company, 207 East 84th Street, New York 28).

6. 6 × 50 mm. serological tubes, *e.g.* Kimble, No. 45060.

Procedure—To the bottom of a 6 × 50 mm. tube are transferred 10 c.mm. of serum[3] and 40 c.mm. of the acid-charcoal suspension (Reagent 3), and the contents mixed by tapping the tube with the finger. (The charcoal suspension in a small vessel is stirred immediately before each pipetting by blowing through the pipette, and the pipette must be filled and emptied rapidly to avoid plugging due to settling of the charcoal.) The tube is capped with a piece of parafilm or a rubber stopper and centrifuged 10

[1] If charcoal gets into the final sample, low values may result. If difficulty is encountered from floating charcoal, 1 volume of 2 per cent gelatin may be added to 10 volumes of the acid-charcoal suspension just before use.

[2] Lowry, O. H., and Bessey, O. A., in preparation.

[3] A convenient method of collecting small amounts of blood for this purpose from the finger has been described (6).

minutes at 3000 R.P.M. A 30 c.mm. aliquot of the supernatant is transferred to another 6 × 50 mm. tube, and 10 c.mm. of the thiourea-dinitrophenylhydrazine reagent (Reagent 1) are added with tapping. The tube is capped as before and incubated for 3 hours at 38°. The tube is now chilled in ice water and 50 c.mm. of ice-cold 65 per cent H_2SO_4 are added, *very thoroughly mixed*, and read after 30 minutes at room temperature in the spectrophotometer at a wave-length of 520 mμ. The sample is transferred to the spectrophotometer with a short Pasteur pipette. Standards and blanks are provided by adding 4 ml. of the acid-charcoal suspension to 1 ml. aliquots of fresh 1 mg. per cent ascorbic acid solution and water, respectively. After centrifuging, 30 c.mm. aliquots are treated in the same manner as the unknowns. Care is taken to avoid floating charcoal, which is more troublesome in the absence of serum. After correction for the blank, the color produced is directly proportional to the concentration of ascorbic acid and, therefore, only the 1 mg. per cent standard is required for routine purposes. In practice, a long series of samples is placed in order in a metal rack and carried through the analysis together.

Once acid has been added to the serum, it may be safely stored several days in the ice box or for several weeks at −20°. If the supernatant acid extract is separated, it may be stored without loss of the ascorbic acid or its derivatives for at least several weeks in the ice box, and presumably indefinitely at −20° (see below). The chief consideration is to prevent evaporation of the small samples. Rubber stoppers have proved most effective. The cut off smaller ends of No. A vial stoppers have been found very convenient for the 6 × 50 mm. tubes. Parafilm is not reliable for the prevention of evaporation from these tubes for more than a short time.

Adaptation to 25 C.mm. of Serum

Apparatus—

1. Constriction pipettes, 25, 75, and 125 c.mm.; also a 100 c.mm. pipette with wider constrictions for pipetting the charcoal suspension.

2. Either the Beckman spectrophotometer or the Coleman Junior spectrophotometer (model 6) with cells and adapter for 0.2 ml. volume. (The adapter is obtainable from Samuel Ash, 3044 Third Avenue, New York 56.)

3. Reagents and tubes as described above.

The procedure is identical to that described above except that all volumes are increased proportionately. To 25 c.mm. of serum in a 6 × 50 mm. tube are added 100 c.mm. of the acid-charcoal suspension. After centrifuging, a 75 c.mm. aliquot is transferred to another 6 × 50 mm. tube and 25 c.mm. of the diphenylhydrazine reagent (No. 3) are added. After incubation 125 c.mm. of 70 per cent sulfuric acid are added.

Adaptation to still larger serum volumes may be effected by simple

multiplication of all volumes, with a not inconsiderable increase in convenience as compared to the original Roe and Kuether procedure.

DISCUSSION

Aside from requiring smaller quantities throughout, the deviations from the Roe and Kuether procedure consist of (*a*) a reduction in the relative quantity of charcoal employed; (*b*) the addition of the charcoal as a suspension in trichloroacetic acid instead of as a dry powder; (*c*) the removal of protein and charcoal in one step by centrifuging instead of first centrifuging the protein, then adding charcoal and filtering; (*d*) combination of the thiourea and dinitrophenylhydrazine reagents; and (*e*) reduction in the strength of the strong sulfuric acid employed.

These changes reduce the number of operations and add greatly to the convenience of the procedure on a small scale. Filtration is scarcely practicable with volumes of the order of 0.05 ml. Although the bulk of the charcoal is easily centrifuged, there is a tendency for some of it to float. This tendency was decreased (so that upon the addition of serum the floating was entirely eliminated) by suspending the charcoal and decanting the floating fraction, and by using less charcoal, since a smaller amount could be shown to suffice for complete conversion of ascorbic acid to dehydroascorbic acid. Removal of protein and ascorbic acid in one step not only reduced the number of manipulations required, but also tended to carry down the charcoal more completely. It was found that identical results were obtained whether the charcoal was added to the serum before the trichloroacetic acid, after the trichloroacetic acid, or separately to the deproteinized serum supernatant. By reducing the strength of the sulfuric acid to be added after incubation from 85 to 65 per cent and chilling this sulfuric acid, the heat produced on mixing was so decreased that the acid could be added all at once, whereas by the original procedure it was necessary to add the sulfuric acid slowly over the course of a minute to prevent charring. A slow addition of this type would be awkward with the small volumes employed here. To compensate for this decrease in acid concentration, proportionately more was used.

Certain analytical considerations which were thought to be of possible significance have been checked and shown to be without influence on the results. These are (*a*) the age of the trichloroacetic acid; (*b*) the size and quality of the glass tubes, whether soft glass or Pyrex; (*c*) the separate addition of the trichloroacetic acid and charcoal in water suspension in any order as compared to addition of the two together; (*d*) the age of the trichloroacetic-charcoal reagent; and (*e*) variation in charcoal concentration from 0.2 to 4 per cent (but with less charcoal there is less tendency for the charcoal to float).

On the other hand, a significant increase in thiourea decreases color development, whereas omission of thiourea increases the blank reading. Decreasing the final acid strength below that recommended decreases the amount of color development.

Comparative Serum Analysis—In Table I are recorded the results of a comparison between the proposed method in which 0.01 ml. of serum is used and the Mindlin and Butler dichlorophenol indophenol method with 1 ml. of serum. The Roe and Kuether micromethod was applied both to individual serum samples obtained from finger blood centrifuged in capillary tubes (6) and to aliquots of a large serum sample obtained by vein. The correlation appears to be quite satisfactory between the two

TABLE I

Comparative Ascorbic Acid Analyses of Blood Serum

The values are given in mg. per cent.

Subject No.	Dichlorophenol indophenol (macro)	Roe and Kuether, vein (micro)	Roe and Kuether, finger (micro)	Bromine,* vein (micro)	Bromine minus Roe and Kuether, vein
1		1.92	1.84	1.97	+0.05
2	1.31	1.34	1.34	1.52	+0.18
3	1.33	1.33	1.35	1.40	+0.07
4	1.01	0.93	1.03	1.18	+0.25
5	1.14	1.11	1.23	1.24	+0.13
6	0.24	0.13	0.20		
7	1.10	1.05	1.12	1.12	+0.07
8	0.32	0.27	0.28	0.28	+0.01
9	1.64	1.63	1.65	1.65	+0.02
10	1.26	1.21	1.28	1.28	+0.07
Average					+0.12

* 1 c.mm. of 2.5 per cent bromine in water.

methods and also between the small finger blood samples and the large venous samples. The values recorded for the small samples are the average of triplicate determinations.

An attempt was made to substitute bromine for charcoal (as the oxidant for ascorbic acid). 1 c.mm. of 2.5 per cent bromine water was added to each tube in place of the charcoal. The procedure worked smoothly with pure ascorbic acid samples, but as is seen in the last column of Table I, there was with serum a tendency for bromine to produce slightly higher results than the other procedures. Evidently charcoal, in addition to oxidizing the ascorbic acid, removes some interfering material. Nevertheless, it may be that for some purposes the bromine procedure would be advantageous, since the use of the somewhat troublesome charcoal reagent

would be avoided. In this case, the thiourea must be added as a separate solution before the rest of the reagent.

Proportionality and Reproducibility—The color development was found to be satisfactorily proportional on the micro scale. For example, with standard solutions equivalent to 0.2, 0.4, 0.8, 1.6, and 3.2 mg. per cent of serum, observed density readings were 0.013, 0.034, 0.069, 0.133, and 0.276, whereas the readings, if proportional, should have been 0.017, 0.034, 0.069, 0.138, and 0.276. Replicate determinations agree satisfactorily. For a series of forty-six sera ranging from 0.3 to 1.4 mg. per cent, analyzed in duplicate, the standard deviation of the individual determination was 0.03 mg. per cent.

TABLE II

Effect of Storage on Ascorbic Acid Values of Blood Serum (Ascorbic Acid Plus Dehydroascorbic Acid)

Per cent ascorbic acid remaining								
Serum (hemolyzed)*	Serum						Serum + acid	
38°	38°		4°		−20°		4°	−20°
1 hr.	1 hr.	4 hrs.	6 days	13 days	6 days	13 days	13 days	13 days
62	95	55	41	6	90	57	96	95

Serum + acid + norit					Acid extract				
25°		4°	−20°		25°	4°		−20°	
2 days	8 days	2 days	2 days	8 days	6 days	6 days	13 days	6 days	13 days
101	145	104	105	102	95†	103†	100	100†	100

*Serum with 2 per cent hemolyzed blood added.

†Stored with 3 per cent metaphosphoric acid. All the rest stored with 4.5 per cent trichloroacetic acid.

Stability—The advantage of the dehydroascorbic acid method from the standpoint of stability is shown by the results of storage tests at various temperatures on serum, serum with added trichloroacetic acid (Table II), serum with added norit and trichloroacetic acid, and extracts of serum made with norit and trichloroacetic acid or metaphosphoric acid. Serum was stored in capillary tubes. For the other samples, 10 c.mm. serum aliquots in 6 × 50 mm. tubes were precipitated with 30 c.mm. of acid with or without added norit and were either stored in these tubes without separating the supernatant or the supernatant was transferred to other 6 × 50 mm. tubes for storage. The samples were analyzed at various times up to 13 days. It is evident that no loss was detectable even at room

temperature with the acidified samples, and even the serum stored at 4° contained considerable amounts of ascorbic acid (presumably as dehydroascorbic acid) after 6 days. Under these conditions only the frozen samples would have contained detectable amounts of reduced ascorbic acid after 2 days; *i.e.*, all but possibly the frozen samples would have given zero values with dichlorophenol indophenol. However, in spite of this considerable stability, some care must be taken in hot weather to keep blood or serum samples cool until they are acidified, since at 38° there is detectable loss in 1 hour and serious loss in 4 hours (Table II). It is further indicated that hemolysis tends to accelerate the process of destruction; hence special care should be taken to keep hemolyzed specimens cool.

It will be noted that for samples stored with norit at room temperature the absorption values increased; evidently the charcoal caused the formation of something which reacts with the Roe and Kuether reagent. The tendency for the charcoal to float is somewhat increased by freezing and the subsequent necessary stirring. This makes it advisable, when samples are to be stored in the frozen state, to separate the supernatant before freezing.

The advantage of being able to store acidified samples until a large number have been collected is obvious. Because of this and the other advantages ascribed to it, the proposed method appears to satisfy the requirements of a micromethod for purposes of nutritional surveys.

SUMMARY

The dinitrophenylhydrazine method of Roe and Kuether has been adapted to the determination of ascorbic acid in 0.01 ml. of serum. The values obtained are in agreement with those obtained by the macro indophenol method of Mindlin and Butler.

Necessary changes in procedure for adaptation to microanalyses appear to represent simplifications applicable to macroanalyses as well.

The micromethod is particularly suited for nutritional surveys owing to (*a*) the ease of obtaining suitable blood specimens, (*b*) the stability of specimens, and (*c*) the convenience for large scale analytical operations.

BIBLIOGRAPHY

1. Mindlin, R. L., and Butler, A. M., *J. Biol. Chem.*, **122,** 673 (1937–38).
2. Butler, A. M., Cushman, M., and MacLachlan, E. A., *J. Biol. Chem.*, **150,** 453 (1943).
3. Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, **147,** 399 (1943).
4. Penny, J. R., and Zilva, S. S., *Biochem. J.*, **37,** 403 (1943).
5. Levy, M., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **21,** 101 (1936).
6. Lowry, O. H., and Hunter, T. M., *J. Biol. Chem.*, **159,** 465 (1945).