CLX. THE ESTIMATION OF REDUCED ASCORBIC ACID IN BLOOD SERUM AND PLASMA.

BY FRANCIS HENRY LASKEY TAYLOR, DORRANCE CHASE
AND JAMES MORISON FAULKNER.

*From the Thorndike Memorial Laboratory, Second and Fourth Medical
Services (Harvard), Boston City Hospital, and the Department
of Medicine, Harvard Medical School, Boston.*

(Received April 25th, 1936.)

THE use of 2:6-dichlorophenolindophenol for the estimation of ascorbic acid was introduced by Tillmans *et al.* [1932]. The specificity of the reaction under suitably controlled conditions has been well established by Harris and Ray [1933], Birch *et al.* [1933] and Johnson and Zilva [1934]. These authors have applied the method variously to the determination of ascorbic acid in urine, tissues and food materials.

The purpose of the present communication is to describe a method for the estimation of small amounts of ascorbic acid in blood. The presence of large amounts of protein in blood serum and plasma renders the direct titration by dichlorophenolindophenol (Tillmans's reagent) impossible. Several methods have been employed for the removal of protein from serum or plasma before titration with this reagent [Mirsky *et al.*, 1935; Roe, 1935; Gabbe, 1934; Emmerie and Van Eekelen, 1934; Farmer and Abt, 1935]. The most widely used method has been precipitation by trichloroacetic acid. However, this reagent is not satisfactory for this purpose because it slowly decolorises the dichlorophenolindophenol indicator, thus making a sharp end-point with the ascorbic acid titration impossible. Farmer and Abt [1935], seeking to avoid the unsatisfactory end-point occasioned by the presence of trichloroacetic acid, resorted to sodium tungstate-sulphuric acid as a protein precipitant, finding that it did not interfere in the titration with Tillmans's reagent. During the past year, working independently, we have employed tungstic acid precipitation in the routine determination of the ascorbic acid content of the blood serum and plasma.

PROCEDURE.

The procedure should be commenced as soon as possible after the blood is drawn to diminish any tendency for cellular breakdown or oxidation of the vitamin. A 2 ml. sample of blood serum (or plasma) is pipetted into each of two 50 ml. centrifuge-tubes to provide for duplicates. 14 ml. of water and 2 ml. of 5 % sodium tungstate are added to each tube. The protein is then precipitated by the addition to each tube of 2 ml. of $N/3$ H_2SO_4 . The contents of the centrifuge-tubes are well mixed and allowed to stand until precipitation is complete and are then centrifuged for 10 min. The supernatant liquid is decanted into Erlenmeyer flasks and acidified with 0.5 ml. of glacial acetic acid. The flasks containing the supernatant liquid from the successive washings of each sample should be kept stoppered in a drawer in the dark until titrated.

The protein precipitate is redissolved in 2 ml. of 5 % sodium tungstate solution by stirring and diluted with water. The amount of water added, together with that used in washing the stirring rod, approximates to 14 ml. The protein is then reprecipitated as before. This re-solution, precipitation and centrifuging is repeated four times; the supernatant liquid being added each time to the previous washings in the Erlenmeyer flasks.

The contents of each of these flasks are titrated with Tillmans's reagent prepared and standardised as given below. 1 ml. of this solution is equivalent to approximately 0.10 mg. of ascorbic acid. The titration is carried out rapidly to a standard end-point, the colour produced by 1 drop of Tillmans's reagent added to boiled distilled water of approximately the same volume as the unknowns, and containing 0.5 ml. of glacial acetic acid in an Erlenmeyer flask similar to that used for the unknown solutions. Whilst the end-point is not fugitive, more consistent results can be obtained by arbitrarily choosing as an end-point the number of ml. of Tillmans's reagent required to produce a colour matching the standard which will persist for 1 min. or more. The "blank" introduced by this method, amounting to not more than 0.005 mg. of ascorbic acid, is then deducted from the value obtained.

Reagents.

1. *Sodium tungstate*. C. P. Sterling Certified, 5 % solution, freshly prepared weekly.
2. *Sulphuric acid*. *N*/3, freshly prepared weekly.
3. *Acetic acid*. C. P. "Baker's Analysed" glacial.
4. *Ascorbic acid*.¹ ("Cevitamic Acid" Merck) Standard solution 1 mg. per ml. (see directions below).
5. *2:6-Dichlorophenolindophenol* (La Motte). 0.2 mg. per ml. (see directions below).
6. *Iodine*. *N*/100 solution in potassium iodide. (Prepared according to Treadwell and Hall [1924].)
7. *Starch indicator*. Prepared according to the method of Treadwell and Hall [1924].

Preparation of standard solution. The standard solution is prepared by dissolving 100 mg. of pure ascorbic acid in 100 ml. of boiled, distilled water. Although the samples of ascorbic acid in the solid form were pure, it was found that the acid was quite rapidly oxidised in solution. The reduced ascorbic acid, remaining in the standard solution, was checked daily by determining its reducing power with *N*/100 iodine solution, using the method of Treadwell and Hall [1924]. The iodine titration values have been found to fall to about one-half in the course of 1 week. The standard ascorbic acid solution should be preserved in a brown glass stoppered bottle and stored in the ice-chest.

Preparation of Tillmans's reagent. 20 mg. of 2:6-dichlorophenolindophenol² are dissolved in 75 ml. of boiling distilled water, cooled, transferred to a 100 ml. volumetric flask and made up to the mark with distilled water. It is then checked daily against the standard ascorbic acid solution. The reagent should be replaced within 2 weeks in order to maintain a sharp end-point. Both Tillmans's reagent and the standard ascorbic acid solution should be protected from light in dark

¹ To Prof. Szent-Györgyi and to the Merck Company, Rahway, N.J., we gratefully acknowledge the gifts of ascorbic acid used in these experiments.

² Sometimes a reddish impurity is found in the commercial product which must be removed by extraction with ether. Alternatively the dye may be prepared quite simply in the laboratory by the method of Cohen *et al.* [1924] as suggested by Bessey and King [1933].

bottles and stored in the ice-box. In all titrations involving the use of Tillmans's reagent the solutions to be titrated are brought to the acid side of Congo red (p_H 4.7) by addition of glacial acetic acid. In the method described 0.5 ml. of glacial acetic acid was usually sufficient for this purpose.

Precipitant. Sodium tungstate-sulphuric acid was adopted as a protein precipitant after many trials of other substances. It was finally used in the method for two reasons: it avoided the fugitive end-point of trichloroacetic acid and the solubility of the protein precipitate in an excess of sodium tungstate afforded a ready way of washing the precipitate free from contained ascorbic acid.

Interfering agents. Under the experimental conditions outlined below the following substances were found not to interfere: phenols, uric acid, amino-acids, urea, creatinine, glucose. As has been reported previously, thiosulphate interferes [Harris and Ray, 1933].

EXPERIMENTAL.

A fairly definite percentage of ascorbic acid is left in the protein precipitate. Several experiments have shown that about 50 % may be recovered without washing, and that after each subsequent washing up to 30 % of the ascorbic acid remaining is occluded. After 4 washings, when small amounts of ascorbic acid are added to the untreated serum, between 89 and 111 % is recovered. Table I gives recoveries obtained from ascorbic acid added to serum or plasma in amounts ranging from 0.0151 to 0.272 mg.

Table I. *Recoveries of ascorbic acid added to blood serum.*

No.	Ascorbic acid present in 2 ml. blood serum mg.	Ascorbic acid added mg.	Total amount ascorbic acid found mg.	Ascorbic acid recovered mg.	Loss or gain mg.
1	0.0386	0.0151	0.0551	0.0165	+ 0.0018
2	0.0316	0.0159	0.0468	0.0154	- 0.0005
3	0.0489	0.0212	0.0696	0.0207	- 0.0005
4	0.043	0.0282	0.0705	0.0275	- 0.0007
5	0.0386	0.0302	0.071	0.0324	+ 0.0022
6	0.0489	0.032	0.079	0.0301	- 0.0019
7	0.045	0.0405	0.0855	0.039	- 0.0015
8	0.045	0.0405	0.0834	0.0384	- 0.0021
9	0.0386	0.0453	0.0875	0.0489	+ 0.0036
10	0.0489	0.0472	0.0960	0.0473	+ 0.0001
11	0.043	0.0564	0.0960	0.053	- 0.0034
12	0.0386	0.0604	0.0938	0.0552	- 0.0052
13	0.034	0.068	0.101	0.067	- 0.001
14	0.034	0.102	0.136	0.102	± 0.000
15	0.043	0.1128	0.145	0.102	- 0.010
16	0.034	0.136	0.167	0.133	- 0.003
17	0.037	0.154	0.174	0.137	- 0.017
18	0.034	0.204	0.244	0.210	+ 0.006
19	0.030	0.216	0.230	0.1995	- 0.016
20	0.034	0.272	0.319	0.285	+ 0.013

In the recently published method of Farmer and Abt [1935] the proteins are also precipitated by sodium tungstate-sulphuric acid. The determination is made on plasma. It has been found that the plasma and serum values agree very well provided that, in addition to the procedure outlined by Farmer and Abt, the proteins are reprecipitated at least three times and washed. Without this precaution, the values were found to be relatively low. The effect of washing and reprecipitation is shown in Table II.

Table II. *Effect of washing and reprecipitation of the protein filtrate on recovery of ascorbic acid from serum and plasma.*

Subject	mg. ascorbic acid per 100 ml. serum (1 washing)	mg. ascorbic acid per 100 ml. serum (4 washings)	mg. ascorbic acid per 100 ml. plasma (1 washing)	mg. ascorbic acid per 100 ml. plasma (4 washings)
Gr.	0.30	0.69	0.34	0.80
McC.	—	1.03	—	0.93
Wa.	—	—	0.18	0.32
Mc.	0.19	0.57	—	—
Sh.	—	0.60	—	0.69
Za.	—	0.87	—	0.79
Gr.	—	0.89	—	1.13
Fa.	—	1.35	—	1.31
Fa.	0.87	1.73	0.82	1.73
Mo.	1.73	2.59	1.59	2.41
Ma.	0.29	0.41	—	—
Gr.	0.70	1.33	—	—
Ch.	0.25	0.57	—	—

Applications. Studies on the output of ascorbic acid in the urine of normal individuals have shown wide variations dependent, to some degree at least, on the intake. The level of ascorbic acid in the blood of normal individuals and patients without infections likewise showed differences roughly parallel with the dietary intake of the vitamin. Table III illustrates values found in a few of a series of individuals representing, from the dietary point of view, a cross section

Table III. *Ascorbic acid content of normal individuals and control patients.*

Subject	Diagnosis	Ascorbic acid content of serum mg. ascorbic acid per 100 ml. serum
Fi.	Normal	2.24
Da.	"	2.03
Cu.	"	1.93
Ta.	"	1.90
Ow.	"	1.70
Fa.	Hypertension	1.69
McG.	Normal	1.63
Pa.	"	1.61
Na.	"	1.60
Fe.	"	1.59
Pl.	Spastic constipation	1.54
He.	Normal	1.50
Fa.	"	1.38
Lo.	Cirrhosis of liver	1.35
Ab.	Taenia saginata	1.25
La.	Hypertension	1.23
Pa.	Anxiety neurosis	1.18
Fl.	Normal	1.04
Wo.	Diabetes mellitus	0.85

of normal individuals in the community. The group contained adults of both sexes and all ages comprising members of the staff and such hospital patients as were free from infections or clinical evidence of dietary deficiency. These determinations were all carried out in the months of July to October, inclusive, in Boston. The average value in 33 such individuals was 1.61 mg. per 100 ml. of serum of reduced ascorbic acid with extremes of 2.43 and 0.83 mg. respectively.

In 10 adult patients with clinical manifestations of scurvy the level of ascorbic acid in the blood was relative low, varying from 0.11 to 0.55 mg. per 100 ml. with an average of 0.245 mg. per 100 ml. (see Table IV).

Table IV. *Blood ascorbic acid in clinical scurvy.*

Case No.	Subject	mg. ascorbic acid per 100 ml.	Case No.	Subject	mg. ascorbic acid per 100 ml.
1	Sm.	0.11*	6	Ta.	0.20*
2	Bn.	0.11*	7	Ba.	0.26*
3	Co.	0.13*	8	O'H.	0.30*
4	Wa.	0.18*	9	Wi.	0.42
5	Jo.	0.19*	10	Re.	0.55
Average					0.245

* The experimental error, being 0.2 mg. per 100 ml., becomes relatively so great with the lower values that it is perhaps preferable to designate any value below 0.4 mg. per 100 ml. as a "trace" rather than to assign a quantitative figure to it.

The effects of the oral and intravenous administration of ascorbic acid on the blood level of this substance are shown in Figs. 1 and 2. The subjects were fasting throughout the experiment. Fig. 1 represents the observations in a normal young adult. The four determinations taken at hourly intervals in a

Fig. 1. Ascorbic acid of blood serum after administration of 1 g. ascorbic acid to a normal subject.
 ↑ Time of administration. — Intravenous. ---- Oral. . . . Fasting control.

Fig. 2. Ascorbic acid of blood serum after administration of 1 g. ascorbic acid to a scurvy patient.
 ↑ Time of administration. ---- Oral. — Intravenous.

fasting condition were constant within experimental error and act as a control for the experiment in this subject. On oral administration of 1 g. of ascorbic acid, the serum level rose from a base line value of 1.57 mg. per 100 ml. to a peak of 2.29 mg. in 1 hour, returning in 5 hours to approximately the initial level. 1 g.

of the vitamin was given intravenously 3 weeks after the previous experiment, at which time the fasting blood serum level of ascorbic acid was 1.21 mg. per 100 ml. 30 min. after injection, the serum value was 3.49 mg. per 100 ml., from which it fell to 2.17 mg. per 100 ml. in 2 hours. At the end of 4 hours the serum value was 2.30 mg. per 100 ml., still significantly higher than the initial level.

Fig. 2 illustrates the findings in a case of scurvy. 1 g. of ascorbic acid was administered intravenously and the blood level of ascorbic acid was determined after 30 min., 1, 1½, 2, 3 and 48 hours. The blood serum level of ascorbic acid rose sharply from an initial level of 0.61 mg. per 100 ml. of serum in 30 min. to 2.58 mg. per 100 ml. of serum. In 1½ hours the value had fallen to 1.24 mg. per 100 ml. and was maintained at this level for at least 48 hours. The curve following oral administration was obtained 7 days later, the patient having been kept on a vitamin C-free diet. The blood level had fallen during this interval to 1.14 mg. per 100 ml. of serum. From this initial level the ascorbic acid content rose very gradually after oral administration of 1 g. of the vitamin in aqueous solution, reaching a level of 1.70 mg. per 100 ml. 3 hours after ingestion. This value was maintained for at least 2 more hours. 24 hours later the ascorbic acid content of the serum had fallen to approximately the initial level.

DISCUSSION.

The transient rise in the blood level of ascorbic acid following administration of the pure substance furnishes indirect confirmatory evidence of the specificity of the method itself. This observation and the finding of relatively low blood ascorbic acid levels in scurvy are in keeping with the suggestion of Farmer and Abt [1935] that the level of reduced ascorbic acid in the blood may be used as a measure of the "nutritional state" of the organism with respect to vitamin C.

The method is presented as a relatively simple and reliable procedure for the estimation of the level of reduced ascorbic acid in blood serum or plasma. As such, it offers a useful tool for the study of the effects of diet and disease upon the metabolism of vitamin C. A report of such studies will be the subject of another communication.

SUMMARY.

1. A method is described for the estimation of reduced ascorbic acid in 2 ml. samples of blood serum or plasma.
2. Recoveries of added ascorbic acid range between 89 and 111 %.
3. In 33 individuals, free from infection or scurvy, the average level of reduced ascorbic acid in the blood serum was 1.61 mg. per 100 ml., with extreme values of 0.83 and 2.43 mg. per 100 ml.
4. Of 10 individuals with scurvy, 8 showed values of reduced ascorbic acid in the blood serum below 0.4 mg. per 100 ml., which is the smallest amount which can be quantitatively determined by the method used. The remaining 2 showed values of 0.42 and 0.55 mg. per 100 ml. respectively.
5. Typical curves are shown illustrating the transient rise in the blood level of reduced ascorbic acid following oral and intravenous administration of the pure substance in a normal subject and in a case of scurvy.

REFERENCES.

- Bessey and King (1933). *J. biol. Chem.* **103**, 687.
Birch, Harris and Ray (1933). *Biochem. J.* **27**, 590.
Cohen, Gibbs and Clark (1924). *Publ. Hlth Rep., Wash.*, **39**, 804.
Emmerie and Van Eekelen (1934). *Biochem. J.* **28**, 1153.
Farmer and Abt (1935). *Proc. Soc. exp. Biol., N.Y.*, **32**, 1625.
Gabbe (1934). *Klin. Wschr.* **13**, 1389.
Harris and Ray (1933). *Biochem. J.* **27**, 303.
Johnson and Zilva (1934). *Biochem. J.* **28**, 1393.
Mirsky, Swadesh and Soskin (1935). *Proc. Soc. exp. Biol., N.Y.*, **32**, 1130.
Roe (1935). *J. biol. Chem.* **109**, LXXV.
Tillmans, Hirsch and Hirsch (1932). *Z. Untersuch. Lebensmitt.* **63**, 1.
Treadwell and Hall (1924). *Analytical Chemistry*, 6th ed. **2**, 551 *et seq.* New York, N.Y.:
John Wiley and Sons.