PERMANGANOMETRIC ESTIMATION OF ASCORBIC ACID.

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(Communicated by Sir J. C. Ghosh, Kt., D.Sc., F.N.I.)

(*Received March 11, 1944.*)

On account of the great importance of vitamin C in animal nutrition, its estimation has assumed considerable importance. The biological method is tedious and not quite convenient for routine estimations. The iodimetric method, which is quite satisfactory for the determination of the vitamin in pure condition, apparently proved inadequate for its estimation in tissue extracts containing other effective reducing substances (which materially interfere with the titer values). The widely used 2 : 6 dichlorophenol indophenol method of Tillmanns, Hirsch, and Hirsch, as modified by Emmerie, Emmerie and Van Eekelen, and Guha and Ghosh, has also its disadvantages. The dye is costly; its solutions invariably deteriorate on standing, and need to be standardised very frequently. Moreover, the pink colour obtained at the end-point fades away rapidly. Borsook, Davenport, Jeffreys, and Warner (1937) studied the rate of reduction of several dyes, e.g., orthocresol indophenol, thionene, indigotetrasulphonate, brilliant alizarin blue etc., by ascorbic acid at different *p*H values. While dehydroascorbic acid did not reduce any of the dyes even in 24 hours between the *p*H limits 2·5 and 4·0, it acquired a capacity for slow reduction of the dyes (excepting alizarin blue) at *p*H 4·5. Above this *p*H the reduction rate of the dyes was much more rapid. The chief point emerging out of the investigations of Borsook *et al.* is the proof that dehydroascorbic acid can become a reducing agent. With the most positive dye used, orthocresol indophenol, the reduction times with solutions of dehydroascorbic acid were always greater than with those of ascorbic acid. Thus from *p*H 2·5 to 4·0 the two times were infinity and less than 1 minute respectively; at *p*H 5·0, 100 minutes and less than 1 minute; and at *p*H 7·0, 6 minutes and less than 1 minute. These differences in reactivity of the reduced and oxidised forms make it possible for determining vitamin C by titrimetric methods with indophenols. This work thus makes clear the need for the control of the *p*H for the titration of ascorbic acid with indophenol. E. G. Ball (1937) studied the titration of ascorbic acid with the dyes methylene blue and 2 : 6 dichlorophenol indophenol. He came to the conclusion that the titration with the methylene blue should be carried out at a *p*H value less than 1·0 to secure quantitative results, while the titration with the indophenol can be carried out at *p*H 4·6. Ball, however, remarks that since such dye stuffs become unstable in more acid regions, the possibility of side reactions under such conditions that may invalidate the use of dichlorophenol indophenol must not be overlooked. The investigations of Borsook *et al.* and Ball demonstrate the need for caution in the use of the dichlorophenol-indophenol dye, though it is much more suitable than the other dyes. The numerous other methods that are proposed such as the Bessonoff phosphomolybdic acid method, the Fujita, Iwataka and Miyata tungstic acid method, the Martini, and Bonsignore methylene blue method, have all their own shortcomings, and generally are not convenient.

In view of these circumstances, we attempted to find a cheaper and more convenient reagent for the estimation of ascorbic acid. We found that under suitable conditions ascorbic acid can be estimated by titration with very dilute potassium permanganate solution using starch-iodide as the indicator.

EXPERIMENTAL.

In all our experiments we used synthetic ascorbic acid of the British Drug Houses Ltd. All the other chemicals used were of Merck A.R. quality.

A weighed quantity of pure ascorbic acid was dissolved in conductivity water (free from traces of copper) and the solution made up to a known volume (10 to 50 mgms. in 50 c.c.) A few c.c. of dilute sulphuric acid (about 2 to 3 c.c. of 2N acid for 50 c.c. solution) were also added to the solution (before making it up to known volume) to retard the spontaneous atmospheric oxidation of the vitamin in solution.

An aliquot part of the ascorbic acid solution was taken in a clean pyrex glass beaker; then 2 c.c. of 2N sulphuric acid, 1 c.c. of 0·5% starch solution, and 0·5 c.c. of 1% potassium iodide solution were added, and the total volume was made up to 20 c.c. by the addition of conductivity water. This was then quickly titrated against standard potassium permanganate (0·01N to 0·001N) run down from a microburette, with constant stirring. A pale blue colour permanent for at least 2 minutes indicates the end-point. A blank determination was carried out without ascorbic acid but under otherwise identical conditions and the volume of permanganate consumed was subtracted from the previous titre value to get the volume of permanganate corresponding to the ascorbic acid taken. Calculations were made on the assumption that one gramme-molecule of ascorbic acid corresponds to two litres of normal permanganate (acid medium).

The permanganate was then replaced by iodine and the process repeated (including the blank determination) to get the iodimetric values. Calculations were made on the basis that one gramme-molecule of ascorbic acid corresponds to two litres of a normal solution of iodine. Care was taken never to expose the iodine solutions, and to preserve them in well-stoppered amber coloured bottles. The strength of the solution was also checked by fresh standardisation whenever necessary. Dilute solutions of iodine deteriorate in strength rapidly.

Following are some of the typical results.

TABLE NO. 1.

Ascorbic acid used contained approximately 10 *mgms. of the vitamin in* 50 *c.c. Varying amounts of this solution were titrated with* 0·001N *solution of iodine and permanganate.*

Volume of ascorbic acid solution taken.	AMOUNT OF ASCORBIC ACID FOUND.		Difference.
	Permanganate method.	Iodine method.	
2 c.c.	0·3882 mgm.	0·3782 mgm.	2·64%
3 c.c.	0·5823 mgm.	0·5673 mgm.	2·64%

TABLE NO. 2.

Ascorbic acid solution used contained approximately 50 *mgms. of the vitamin in* 50 *c.c. Varying amounts of this solution were titrated with* 0·01N *solution of iodine and permanganate.*

Volume of ascorbic acid solution taken.	AMOUNT OF ASCORBIC ACID FOUND.		Difference.
	Permanganate method.	Iodine method.	
2 c.c.	2·016 mgms.	2·014 mgm.	0·1%
3 c.c.	3·033 mgms.	0·030 mgm.	0·1%

We have carried out numerous estimations. While working with 0·001N solution of permanganate, the deviations from the iodimetric method varied from 1 to 3%; while working with 0·01N solution of permanganate, the deviations varied from 0·1 to 0·3%.

With a view to extending the applicability of the new method to the estimation of vitamin C in extracts from plant and animal tissues, we investigated the influence of some substances (which are commonly found in tissues) on the titration of ascorbic acid by permanganate. In these experiments we used 1% solution of the pure chemicals.

A solution of ascorbic acid was titrated both in the presence of the added substance and in its absence in the manner already described, and the titer values compared. Any difference greater than the possible experimental error should be attributed to the interfering effect of the added substance on the method. It was found that glucose, fructose, and succinic, lactic, malic, citric, and tartaric acids do not interfere with the method (in concentrations up to 10 c.c. of 1% solution in 20 c.c. total volume).

Discussion.

It will be seen from the results recorded in the foregoing tables that in the titration of ascorbic acid by very dilute potassium permanganate (N/100 or less) in the presence of starch-potassium iodide indicator the end-point is secured as soon as one gramme-molecule of ascorbic acid consumes a quantity of permanganate equivalent to an atomic proportion of oxygen. This indicates that the oxidation stops with the formation of dehydroascorbic acid. The experimental conditions described here are different from those of Herbert, Hirst and co-workers (1933), who titrated ascorbic acid directly (without the iodide indicator) with relatively concentrated solutions of potassium permanganate. They found that the permanganate was decolorised almost instantaneously until the equivalent of about 1·3 atoms of oxygen had been added. A slower reaction followed which ended somewhat indefinitely when permanganate equivalent to 2 atoms of oxygen had been used up. Thereafter, the reaction proceeded still more slowly without a definite end-point. The arrest at the end-point corresponding to 1·2 atoms of oxygen is caused by a slight overlapping of the first and second stages of oxidation, the primary oxidation product being readily attacked by permanganate. The essential differences between the experiments of Herbert *et al.* and of the present authors are: (1) the employment of relatively concentrated solutions by Herbert *et al.* while we used very dilute solutions, and (2) the use of potassium iodide-starch indicator in our experiments, while Herbert *et al.* resorted to direct titration. Under our conditions the permanganate appears to interact first with the hydriodic acid resulting in the liberation of iodine. The iodine thus liberated will then interact with the ascorbic acid in the well-known manner. This idea is supported by the observation that there is always the appearance of an incipient blue colour immediately a drop of permanganate solution from the burette touches the solution in the beaker; the blue colour, however, disappears on stirring till the end-point is reached, when it becomes permanent. This effect is more prominent in titrations with 0·01N solutions of permanganate. As soon as all the ascorbic acid is converted into dehydroascorbic acid, further addition of permanganate cannot effect the oxidation of dehydroascorbic acid, as immediately on the addition of permanganate, iodine is liberated which, being incapable of oxidising dehydroascorbic acid in the acid medium, produces the permanent blue colour with the starch which is indicative of the end-point of the titration. Thus our permanganometric method of estimation bears a close resemblance to the iodimetric method as in both the methods iodine functions as the direct oxidising agent.

The special advantage of the permanganate method over the iodine method is that potassium permanganate solutions do not suffer changes in concentration for a long time if kept in well-stoppered amber coloured bottles, while iodine solutions speically in dilutions like 0·001N (employed in the estimation of vitamin C) are apt to deteriorate continuously due to the evaporation of iodine necessitating fresh standardisation at every stage. This involves great inconvenience in routine experiments. Permanganate is in addition a cheaper reagent than iodine.

Estimation of vitamin C in plant tissues by the 2 : 6 dichlorophenol indophenol, the iodine, and the permanganate methods :—

The dye method of estimating vitamin C having become very common, no one seems to have paid serious attention to the possibility of the estimation of ascorbic acid in tissue extracts by the iodine titration method after elimination of interfering substances by treatment with mercuric acetate. A survey of the literature has shown that the substances which interfere in the iodine titration, e.g. glutathione, ergothionene, tannins, etc. also interfere in the dye titration. Hence the recommendation of Emmerie, and Emmerie and Van Eekelen to remove these by the addition of mercuric acetate. It seemed to be of considerable interest to try the iodine titration method also after a similar treatment. The few persons that have tried this reported higher values for the vitamin by the iodine method than those obtained by the dye method. But it must be mentioned here that it is necessary to carry out the iodine titration with particular care and having due regard to—

(1) the sensitivity of the starch-iodide indicator,
(2) the stability of the very dilute solutions of iodine of the order of N/1000, and
(3) the standardisation of iodine solution with very dilute solution of sodium thiosulphate which is usually standardised making use of the reaction between dichromate and hydriodic acid which involves many difficulties.

The sensitivity of the starch indicator depends on (1) the iodine concentration, (2) the iodide concentration, (3) the acid concentration, (4) the concentration of other substances. In titrations with iodine solutions it is advisable to have a concentration of at least 2 c.c. of 0·25% starch per 20 c.c. and a sufficiently high concentration (not too high) of iodide and acid to obtain accurate results.

In routine estimations of vitamin C in tissue extracts by the iodine method workers might not have been careful enough to maintain the necessary iodide and mineral acid concentration with the result that a little too much of iodine is taken for the production of the blue colour. Moreover, they might not have subtracted the amount of iodine actually consumed in producing the blue colour with the indicator by carrying out a blank determination in the absence of ascorbic acid but under otherwise identical conditions.

Again, it has been observed that very dilute solutions of iodine deteriorate in strength on keeping for even a few hours. Hence, unless one standardises them just before titrating the vitamin C extracts, one is likely to consume more iodine and get spuriously high values.

Further, the reaction between dichromate and hydriodic acid is slow, specially at the low dilutions of the dichromate (0·01N to 0·001N) employed for the standardisation of correspondingly dilute solutions of thiosulphate. Unless one takes adequate precautions to overcome the several difficulties encountered (cf. Viswanadham, C. R., and Gopala Rao, G., 1942) one is likely to get erroneous values for the strength of the hypo solution.

Thus it will be seen that the high values for the vitamin in tissue extracts by the iodine method previously reported might have been due to the difficulties mentioned above.

In the following experiments, we made simultaneous estimation of vitamin C extracts by the three different methods taking all the necessary precautions.

Experimental.

5 to 10 gms. of the plant material was washed well with conductivity water (free from traces of copper), cut into small pieces in a porcelain dish, and a convenient portion of this was weighed out into a tared beaker containing 25 c.c. of trichloracetic acid (20%). The weight of the sample was noted by difference. The contents of the beaker were transferred into a clean glass mortar, and the beaker rinsed well with conductivity water. The material was then ground well with quartz sand (free from Fe^{+++} etc.). The liquid extract thus obtained was decanted, and the sand washed well. The original extracts and the washings were mixed and centrifuged in an electric centrifuge at 2,500

revolutions per minute. The entire centrifugate was made up to 100 c.c. in a measuring flask. A known volume of this extract (25 to 50 c.c.) was pipetted out into a clean beaker, 0·5 to 2 c.c. of mercuric acetate (20%) added, and again centrifuged. This removes reducing substances other than ascorbic acid as already mentioned. The clear liquid was treated with hydrogen sulphide to remove the excess of mercuric acetate as sulphide. The time between the treatment with hydrogen sulphide and the first addition of mercuric acetate should not exceed 10 minutes. After the mercuric sulphide was precipitated by rapidly passing hydrogen sulphide, the liquid was again centrifuged to sediment the sulphide. The clear centrifugate now obtained was saturated with hydrogen sulphide and left in contact with it overnight in a refrigerator. The hydrogen sulphide reduces any dehydro ascorbic acid extracted from the tissues or otherwise formed. The excess of hydrogen sulphide was removed by first evacuation with the water pump, and later with the Cenco hyvac pump using a wash bottle containing lead acetate solution to absorb hydrogen sulphide, and anhydrous calcium chloride towers to absorb the moisture. Finally a rapid stream of carbon dioxide was passed through the extract to remove the last traces of hydrogen sulphide. Care should be taken completely to eliminate the last traces of hydrogen sulphide, because hydrogen sulphide also reduces the reagents employed for the vitamin C estimation. This extract was diluted to 100 c.c. and an aliquot portion taken for the volumetric estimation, the volume selected depending on the amount of ascorbic acid present.

(1) *The dye method.*—The extract was accurately pipetted out into a small beaker containing 2 c.c. of 2N sulphuric acid and titrated with M/250 freshly prepared 2 : 6 dichlorophenol indophenol solution run down from a micro-burette, with constant stirring, the end-point being indicated by the persistence of a pink colour for at least 30 seconds. A correction was applied for the small quantity of dye solution required to give a similar colour shade in a blank titration (without ascorbic acid). The dye solution was standardised against a solution of synthetic ascorbic acid which in its turn was standardised against iodine solution of known strength.

(2) *The iodine method.*—An aliquot portion of the extract was pipetted into a beaker containing 2 c.c. 2N sulphuric acid, 0·5 c.c. 1% potassium iodide, and 2 c.c. 0·5% starch, the total volume made up to 20 c.c., and titrated against a standard solution of iodine (N/250) run down from a microburette, with constant stirring, the end-point being indicated by a blue colour permanent for at least 2 minutes. A correction was applied for the small amount of iodine required to produce the same shade of colour in a blank titration (without ascorbic acid).

(3) *The permanganate method.*—Same as (2) replacing iodine by potassium permanganate.

The results of these experiments are recorded below.

Name of the plant material.	Ascorbic acid in mgms. per gm. of material.		
	Dye method.	Iodine method.	Permanganate method.
Indian gooseberry (unripe) [16-2-1942]	2·636	2·580	2·740
Indian gooseberry (ripe) [27-3-42]	1·349	1·410	1·530
Tulasi (white) *Ocimum vitosum*	0·4745	0·4798	0·4978
Lime pickles	0·2940	0·2730	0·2940

It will be seen from the results that there is fair agreement between the three different methods. The results given on the variation of vitamin C content of Indian gooseberry fruit perhaps lead to the conclusion that the ascorbic acid content first increases slightly and then suffers a considerable decrease as ripening progresses.

Summary.

(1) A modified method for the estimation of vitamin C involving the use of potassium permanganate has been proposed.

(2) A comparative study of the dye method, the iodine method, and the permanganate method for the estimation of vitamin C in plant sources was undertaken. The results tend to show that the iodimetric method and the permanganometric method can be used with accuracy for the estimation of vitamin C in natural sources, after the usual treatment with mercuric acetate etc., assuming, as is generally done, that the reducing substance is identical with vitamin C. The high values obtained by workers previously for the vitamin by the iodine method have been sought to be explained with reference to the various difficulties underlying the method.

The authors are deeply indebted to Dr. G. Gopala Rao, Reader in Chemistry, Andhra University, for suggesting the problem and for his kind interest in the progress of this work. Their thanks are due to Mr. T. V. Subba Rao, M.Sc., for assistance in this investigation. They are also grateful to Prof. Sir J. C. Ghosh, Director, Indian Institute of Science, for the keen interest he has evinced in this work.

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