CCXLI. THE BIOLOGICAL AND TITRIMETRIC DETERMINATION OF VITAMIN C.

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(Received August 25th, 1934.)

THE present communication deals with a comparison of the biological and titrimetric methods of determination of vitamin C, the materials examined being lemon juice, dried hips and ascorbic acid.

The biological determination of vitamin C.

The experiments were carried out by the protective method. Guinea-pigs, weighing about 200–250 g., were used, since these animals react very readily to vitamin C deficiency. The animals were put on a diet which was vitamin C-free but complete in other respects; to this diet a certain amount of the substance to be examined was added daily.

Working with batches of two or three animals, each of which received different additions, it was possible to determine the smallest quantity which just protected the guinea-pig from scurvy.

As the estimate is rather approximate in the absence of knowledge of the degree of latent scurvy in animals showing a negative gross result, it is important to ascertain the developmental degree of vitamin C-avitaminosis by microscopic examination of the root of the incisor of the guinea-pig before the animal shows any symptoms of scurvy.

Höjer [1924] proposed a method of estimation of vitamin C which depends on the fact that C-avitaminosis in guinea-pigs causes specific changes in the histological structure of the tooth manifested by degeneration of the odontoblasts with subsequent disturbance in the formation of dentine.

It is possible in longitudinal sections of the normal tooth to observe that the odontoblasts normally grow longer until fully developed and then atrophy and finally disappear.

Parallel with the development and degeneration of the odontoblasts runs the formation of the dental bone, the latter being fully developed when the odontoblasts in the crown of the tooth disappear.

In the case of C-avitaminosis the odontoblasts degenerate at an earlier stage, and with decreasing vitamin C supply the degeneration can be observed still closer to the root, finally occurring in the deeper part of the root itself.

Since the degeneration which the odontoblasts undergo in C-avitaminosis cannot be distinguished morphologically from the physiological degeneration, histological examination must be made of sections where normally only fully developed odontoblasts can be found, i.e. of sections through the fang part of the tooth.

According to Höjer the section ought to be taken horizontally on the lower jaw through the middle part of the front molar; this plane of sectioning is likely to hit the fang of the incisor horizontally also, and the measurement of vitamin C is then determined by the minimum amount which is just capable of preserving the normal histology of this particular region of the tooth.

A classification may also be carried out. Based on the histological conditions Key and Elphick [1931] have constructed a scale comprising five groups calculated for an experimental

period of 15 days starting from the picture corresponding with no protection (series 0) and ending with the normal (series IV). The experiments are carried out as protective experiments. To ensure that the guinea-pigs at the beginning of the experiment are without latent scurvy and at the same time without significant stores of vitamin C, the animals are fed for 3 weeks on Key and Elphick's vitamin C-free diet with a daily addition of 50 mg. dried hips, this diet having proved to provide exactly the sufficient amount of vitamin C.

Guinea-pigs prepared in such a way, and as far as possible of the same weight, are put on experiment for 14 days, being fed on the above-mentioned diet with additions chosen for the experiment, the histological examination is made as before stated in the topographical sections according to Höjer and conforming to the scale of Key and Elphick.

On the 14th day the animals are killed. The lower jaw is split between the incisors; the lower jaw halves are taken out and adhering muscular tissue is removed. The tissue is fixed during 8–10 days in Orth's fluid and washed in running tap-water for 24 hours. It is decalcified by treatment for 3 days with 20 parts 5 % nitric acid + 3 parts 3 % iron-alum. The tissue is then placed in 5 % potash-alum for 24 hours and washed in running water for 24 hours. It is finally dehydrated and paraffin sections are stained with alum-haematoxylin-eosin.

The results obtained with dried hips, the juice of Messina lemons and ascorbic acid are shown in Table I. It was found that 1.2–1.5 ml. lemon juice, 50 mg. dried hips and 0.7 mg. ascorbic acid are equivalent, *i.e.* they have the same biological effect since these doses are able just to preserve the normal structure in Höjer's section. Lemon juice accordingly contains 0.4–0.6 mg. ascorbic acid per ml. and dried hips contain 1.4 % ascorbic acid.

Table I. *Showing degree of protection against scurvy, measured on the Key and Elphick scale, afforded by various substances.*

Preparation	mg.	Key and Elphick scale	Preparation	ml.	Key and Elphick scale
Ascorbic acid	0.6	III–IV	Lemon juice	1.5	III
"	0.6	III	"	1.5	IV
"	0.9	IV	"	2.0	IV
"	0.9	IV	"	2.0	IV
"	1.2	IV	"	2.5	IV
"	1.2	IV	"	1.0	III–IV
"	0.6	III	"	1.0	III–IV
"	0.6	III	"	1.5	IV
"	0.6	II–III	"	1.5	IV
"	0.9	IV	"	2.0	IV
"	0.9	IV			
"	0.9	IV		mg.	
"	0.64	II–IV	Dried hips	30	II–III
"	0.64	III	"	30	II–III
"	0.8	IV	"	40	III
"	0.8	IV	"	40	III
"	0.64	IV	"	50	IV
"	0.64	III–IV	"	50	IV
"	0.8	IV	"	60	IV
"	0.8	IV	"	60	IV
"			"	80	IV
"			"	80	IV

Titrimetric determination of vitamin C.

To determine vitamin C titrimetrically 2 : 6-dichlorophenolindophenol can be used as combined oxidant and indicator [Tillmans *et al.*, 1932]. These workers suggest the use of a solution of 0.001 *M* 2 : 6-dichlorophenolindophenol at a reaction of p_H 5–6. By this method an intense blue end-point is secured, while a weaker purple end-point is obtained at a more strongly acid reaction. The latter end-point is utilised by Harris and Ray [1933] in order to circumvent

the danger of other reducing substances influencing the reaction. Harris and Ray also suggest an "inverse" titration, *i.e.* admixture of the solution to be tested with a previously measured 0.01 *M* indicator solution.

We preferred after a thorough trial to follow Tillmans's directions, using however a somewhat stronger indicator solution. With the relatively concentrated ascorbic acid solutions obtained from hip extracts and lemon juice a considerably stronger indicator concentration than 0.001 *M* can be used with advantage. To neutralise free acid 50 % sodium acetate was added. The destruction of the indicator itself at a too strongly acid reaction is in favour of the above-mentioned technique. At alkaline reactions the reduction takes place too slowly and the vitamin C is too easily oxidised by the oxygen of the atmosphere; this error can be avoided by working at p_H 5-6. Furthermore it is advantageous to employ the easily discernible blue end-point.

The "inverse" titration of Harris and Ray can only be used with stronger ascorbic acid solutions, because the determination of the moment when the colour disappears becomes difficult at high dilutions. It has been found to be more certain to determine the appearance of a colour than its disappearance, and therefore the usual method of titration was preserved. At the indicated p_H the titration can be carried out without running the risk of autoxidation of the ascorbic acid but for additional security we avoided too strong daylight and did the titration as fast as possible. The ascorbic acid used for the purpose was a pure preparation, placed at our disposal by Prof. Szent-Györgyi. Its purity was confirmed by iodimetric titration in our laboratory to be 100 %.

Hip extract. To obtain this extract of hips a small amount of dried hip-powder (produced by the Vitamin Laboratory, Skelskor) was boiled for 10 minutes under a vigorous stream of nitrogen in a proper amount of water, a few drops of sulphuric acid being added. The loss of water by evaporation was made good. After refrigeration the mixture was filtered *in vacuo* from a separating-funnel from which the air had been displaced with nitrogen or carbon dioxide. The separating-funnel was closed by a glass stopper and its stem passed through a tight-fitting rubber stopper to discharge into a conical filter-funnel which was contained in a previously evacuated desiccator. An aliquot of the filtered solution was then titrated with 2 : 6-dichlorophenolindophenol with addition of sufficient sodium acetate to produce a blue end-point.

For 0.345 g. hip-powder were used 80 ml. water. 10 ml. were titrated and reduced 1.45 ml. 2 : 6-dichlorophenolindophenol, of which 2.80 ml. had been ascertained to be equivalent to 1 mg. ascorbic acid.

It thus follows that 0.345 g. of hips is equivalent to $8 \times 1.45 \text{ ml.} = 11.60 \text{ ml.}$ indicator solution supposing all vitamin C has been extracted. Consequently 12.0 mg. or 1.20 % ascorbic acid were contained in 1 g. hips.

After careful filtration a second boiling-out by the same method was performed, but the filtrate therefrom appeared to be incapable of reducing the indicator solution.

From time to time similar extractions of hip-powder have subsequently been made. The second extraction never gave any titration value; it is safe to assume therefore that all vitamin C was removed by the first extraction. It must however be noted, that it is somewhat difficult to obtain constant values. The above-mentioned represents the highest value obtained.

There seem to be only two possible explanations of the variation: either an incomplete extraction of the hip-powder or a destruction of part of the vitamin C in spite of all precautions. It is reasonable then to take the highest attained value as representing the content of vitamin C in hip-powder obtained by titration,

supposing of course that no other substances capable of reducing 2 : 6-dichlorophenolindophenol are contained in the extract.

However, as is evident from the above-mentioned biological experiments, the actual content of ascorbic acid is somewhat higher than the maximum value obtained by titration; the latter must consequently be taken in practice to represent a minimum value.

Lemon juice. Titration of ascorbic acid in lemon juice was performed with two different shipments of lemons. The experiment with guinea-pigs lasted for 14 days and samples of the juice were titrated daily.

For each titration 15 ml. of juice were used; after expression 6 ml. 10 % normal lead acetate solution were added, followed by 9 ml. 20 % sodium sulphate to precipitate excess of lead.

After filtration, the perfectly clear solution was titrated with 2 : 6-dichlorophenolindophenol, which, as in the case of the previous titration of hip extract, had been standardised against pure ascorbic acid. The titration value was calculated on the basis of the original undiluted juice.

In all 14 titrations were carried out, which on an average gave a content of 0.39 mg. ascorbic acid in 1 ml. of lemon juice. This first experiment lasted from Nov. 29th to Dec. 13th, 1933, inclusive; in the second experiment from March 12th to 25th, 1934, inclusive the juice on each day was similarly titrated, and these titrations gave on the average 0.56 mg. ascorbic acid in 1 ml. of lemon juice.

It was evident that the juice used in this last experiment was considerably richer in vitamin C than the juice used in the preceding experiment. It was also evident from the appearance of the lemons that the latter were far riper than the lemons from the preceding experiment.

SUMMARY.

It is evident from these results that for the guinea-pig 0.64 mg. ascorbic acid corresponds very nearly to the necessary addition of vitamin C to a diet deprived of all its vitamin C beforehand, while 0.8 mg. is thoroughly satisfactory. The true value is then to be found between these, *i.e.* about 0.7 mg.

Correspondingly the necessary amount of lemon juice in the first experiment lies between 1.5 and 2 ml., in the second experiment near to 1 ml. Approximating these values at 1.75 ml. and 1.25 ml. respectively we obtain 0.4 and 0.55 mg./ml. as the contents of ascorbic acid in the juices.

For dried hips the necessary minimum lies between 40 and 50 mg. Taking this to be 45 mg. it is evident that dried hips contain 1.5 % ascorbic acid.

A comparison between the results of the biological determination and those found by titration shows fairly good concordance; only in the case of dried hips is a slightly higher content of ascorbic acid found by biological assay, probably because the preparation of the solution for titration fails to extract the whole of the ascorbic acid.

The analyses of the lemon juice have revealed considerable variation in the vitamin C content. Consequently lemon juice has to be considered unsuitable as a vitamin C standard.

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