CCLXXIV. STANDARDISATION OF THE ANTI-SCORBUTIC POTENCY OF ASCORBIC ACID.

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THE results to be described in the present paper confirm conclusions given in an earlier note [Harris and Ray, 1933]. We reported there that ascorbic acid—specimens derived from two different raw materials were examined—had an activity such that 2 mg. were equivalent to 3 cc. of orange juice. This would appear to indicate an activity of 15 International Units per mg., since orange juice is generally assumed to have an activity about equal to that of lemon juice, and the ascorbic acid contents as estimated by titration are similar. To confirm the original finding, and obtain a value of as highly precise a nature as possible a rather more elaborate test was then carried through and concluded in May 1933, and the same result was again obtained. A note by Key and Morgan [1933] which has just appeared gives a somewhat lower value than ours—*viz.* 7.4 instead of 15. Our results, although they are based on the use of orange juice as a secondary standard and not on a direct comparison with lemon juice as primary standard (which, however, may vary considerably in potency, as shown below), have the advantage of being founded on more extensive statistical data and involve also a more direct comparison with standard material fed simultaneously at the identical levels of potency, and we think it may be of interest to publish the details.

EXPERIMENTAL.

The ascorbic acid was a very pure specimen from paprika, several times recrystallised and giving an iodine titration of over 99 % of the theoretical. The tooth structure method [Höjer, 1924; 1926; Key and Elphick, 1931] was used, the general procedure being the same as previously indicated [Harris and Ray, 1932]. Ascorbic acid was fed to groups of guinea-pigs at three different levels 1.5 mg., 2.0 mg. and 2.5 mg. per day. (The doses were weighed out daily and dissolved in air-free water immediately before administration, the dilution being such that 1 mg. of ascorbic acid was present in 2 cc. of water. The main specimen was kept in an atmosphere of CO₂ and stored dry at 0°.) Simultaneously three different levels of orange juice were fed to controls, at what were adjudged, on the basis of the earlier tests, to be corresponding levels of activity, *viz.* 2.25 cc., 3 cc. and 3.75 cc. Six animals were included in each group. These particular levels of activity were chosen as giving a fairly full degree of protection, a condition which we find to give more consistently reproducible and sensitive results than at lower partial levels of protection. The animals were killed after 15 days and cross-sections of the incisors prepared in the usual way and assessed for degree of protection.

Our use of orange juice as a secondary or derived standard in place of lemon juice used direct as primary standard may be criticised, but we believe it to have some advantages. Previous papers both from the Pharmaceutical Laboratory and this laboratory have recorded that more constant and easily reproducible results were obtained with orange juice than with lemon juice, apart of course from the additional consideration that the animals take it better, and there is no need to introduce a decitration process when feeding the larger doses which are needed at top levels in the tooth protection method. Confirmation has recently been forthcoming in the report of Bennett and Tarbert [1933] that lemon juice may indeed vary most erratically in potency, the vitamin C content as measured by titration in poorer specimens being little more than about half that of a "good" specimen, whereas orange juice tended to be more constant with a value about equal to or very slightly above that of a "good" lemon juice. It is obvious therefore that the result obtained in any vitamin C determination will depend on the interpretation attached to the standard "lemon juice," whether this is taken to imply lemon juice of a "good" quality, of "low," or of "average" quality (if such can be defined). Key and Morgan have also referred to the apparent variation in potency of lemon juice.

RESULTS.

The values found are given in Fig. 1 and for the third determination in Table I. When these are plotted it is seen that the readings for orange juice and ascorbic acid are equated when the scales for orange juice and ascorbic acid respectively are fixed at 3 cc. of the former against 2 mg. of the latter. This conforms with the earlier findings. It coincides, also, with the amount of

Fig. 1.

○ ascorbic acid; × orange juice.

Table I.

Amount of material fed per day	Degree of protection afforded							Mean
	Individual animals							
Ascorbic acid, 1.5 mg.	2.5,	2,	2.5,	2	, 3	, —		2.5
2 mg.	4	, 3,	3.5,	3	, 4	, 3.5		3.5
2.5 mg.	4	, 4,	4	, 4	, 4	, —		4
Orange juice, 2.25 cc.	2	, 4,	2	, 3	, 2.5,	—		2.7
3 cc.	2.5,	4,	4	, 3	, 3.5,	4		3.5
3.75 cc.	4	, 4,	4	, 3.5,	3.5,	—		3.8

ascorbic acid in an average specimen of orange juice as found by titration in our experience (*viz.* 0.6 to 0.7 mg. per cc.). It represents a value of about double Key and Morgan's.

COMMENTS.

In Key and Morgan's determination, one level only of the lemon juice standard was fed in each experiment and that was administered at levels widely divergent from those of the "unknown" (especially in Exps. 1 and 2). In order to bridge the gap recourse was had to a standard reference curve relating degree of protection in any experiment to the relative amount of vitamin which is needed to produce it. Although we feel that it is in fact a safer procedure to obtain several points on the curve for each determination, and to have one point, at least, for the unknown always lying between two reasonably adjacent values for the standard (or *vice versa*), it nevertheless seems likely that the difference between our result and that of Key and Morgan is mainly attributable to another cause—*viz.* the relatively high rating given by them to their single lemon juice standards. There is quite good agreement between Key and Morgan's values and our own as to the actual degrees of protection afforded by the varying doses of ascorbic acid. Their results agree with ours in showing that 2 mg. are about the minimum dose for giving approximately full tooth protection in a 15-day test. Turning now, however, to Key and Morgan's readings for lemon juice, it would appear that the potency was assessed at double the value that might have been anticipated. Thus, 1.25 cc. of lemon juice gave the same protection as 2.30 cc. of the orange juice of the standard reference curve, and 1.0 cc. of lemon juice as 2.21 cc. of orange juice; and their combined results [Fig. 2, Key and Morgan, 1933] would imply that full protection would be given by as little as 1.48 cc. of lemon juice. This may be compared with the more usually accepted finding that about 3.0 cc. (*i.e.* double the amount) of orange or lemon juice are in fact normally needed to give full protection. One explanation of the apparent divergence between their results and ours, then, would be that they were dealing with a lemon juice of exceptionally high potency, or alternatively that our orange juice was relatively low; or both. This is not necessarily the correct explanation however; indeed orange juice tends on the whole to be somewhat more potent and not less potent than lemon juice. It could be equally well argued that the great variation between the individual animals in Key and Morgan's small number of lemon juice groups could have given rise to an illuively high average. Thus with 1.25 cc. of lemon juice the individual scores ranged from 1.5 to 4.0 and the average was put as 3.2; with 1.0 cc. of lemon juice they ranged from 0.5 to 4.0, with average at 3.1. If there had been a variation in 1 or 2 animals, within the actual range found in the group as a whole, it could easily have reduced the average score to the anticipated value. (In the case of

Table II. *Ascorbic acid content of lemon juice and orange juice.*

I. Lemon juice		
Day	Specimen number and description	Ascorbic acid content as determined by titration mg. per cc. Corresponding calculated activity of ascorbic acid units per mg.
1st	1 (small)	0.515 19.4
	2 (large)	0.407 24.6
	3 (small)	0.583 17.2
	4 (very large)	0.183 53.2
	5 (medium large)	0.380 26.3
	6 (large)	0.44 22.7
	7 (large)	0.41 24.4
	8	0.475 21.1
	9	0.506 19.8
	1st day, average of 9 specimens	0.43 23.3
2nd	10	0.556 18.0
	11 (small and withered)	0.684 14.6
	12 (large and fresh)	0.354 28.3
	13 (small and withered)	0.661 15.1
	14 (very small and withered)	0.694 14.4
	2nd day, average of 5 specimens	0.59 17.0
3rd	15	0.373 26.8
	16	0.381 26.3
	17	0.669 15.0
	18	0.339 29.5
	19	0.472 21.2
	20	0.342 29.2
	3rd day, average of 6 specimens	0.43 23.3
	Three days, average of 20 specimens	0.47 21.3
4th	A composite specimen = mixed juice of 10 lemons	0.47 21.3
II. Orange juices		
1	1 (South African)	0.570 17.5
	2 "	0.570 17.5
	3 (Brazilian)	0.480 20.8
	4 "	0.620 16.1
2	5 (South African)	0.806 12.4
	6 "	0.909 11.0
3	7 "	0.628 15.9
	8 "	0.833 12.0
	9 (Brazilian)	0.757 13.2
	10 "	0.585 17.1
4	11 "	0.586 17.1
	12 "	0.510 19.6
5	13 "	0.533 18.8
	14 "	0.476 21.0
	Average of 14	0.63 15.8

Table III. *Ascorbic acid content of orange juice.*

As determined by titration	...	...	0.63 mg. per cc.
As calculated from biological assay	...	...	0.66 "

the first experiment, with 0.75 cc. of lemon juice and 0.3 mg. of ascorbic acid, there is difficulty in extracting a relationship, since the degree of protection was only very partial in both cases and the lemon juice was being fed at over 3 times the level of potency of the ascorbic acid, making comparison very insecure¹.)

In conclusion we would allude again to the fact that our value coincides with the actual ascorbic acid content of average orange juice or good lemon juice, as determined by chemical titration. The same correlation holds equally for a number of other sources [Harris and Ray, 1933]. This seems a strong argument in its favour. Key and Morgan's value of 7.4 units per mg. would indicate an ascorbic acid content of 1.35 mg. per cc. for lemon juice, a value which would appear difficult to reconcile with the fact that in our experience the reducing titre rarely rises above a value equivalent to about 0.7 mg.

Titration results on a series of lemons and oranges purchased locally from retailers in October are given in Table II. The average ascorbic acid content of the lemon juice (based on determinations on 20 separate lemons, and on the mixed juice of a further 10) was appreciably lower than that of the oranges examined at the same time (*viz.* 0.47 mg. per cc. for lemon juice, as against 0.63 for orange). It must be noted, however, that individual lemons show striking fluctuations in potency, the mean deviation from the average value of 0.47 mg. per cc. being as great as ± 0.11 . (It was observed that higher values were generally given by lemons which were small and shrunk in appearance, and lower values by large "juicy" ones—as though the juice became more concentrated by evaporation during the storage process.) The figure for the orange juice is in agreement with our biological determination of the relative activities of ascorbic acid and orange juice (Table III) although we have found that different varieties of orange juice may also vary considerably in potency. If lemon juice of the "average" quality represented by these specimens is taken as standard, the calculated antiscorbutic activity of ascorbic acid works out at the somewhat higher value of 21 units per mg., as against the value 15 units per mg. compared with a "good" lemon juice equal in potency to that of the orange juice used in our tests.

Since "lemon juice" without further specification has already been fixed as International Standard, and in our opinion the antiscorbutic activity of lemon is equivalent to its ascorbic acid content as estimated by titration, the final step in transferring the standard from lemon juice to ascorbic acid will have to involve a decision as to the exact quality of lemon juice which may be held to represent a fair and normal specimen.

SUMMARY.

1. Assays by the tooth protection method, using large numbers of guinea-pigs, and with ascorbic acid and orange juice always fed simultaneously at various identical levels of potency, show consistently that the activity is such that 2 mg. of ascorbic acid are equivalent to 3 cc. of orange juice, or the minimum dose for full tooth protection (under the conditions of the test) is 2 mg.

¹ In our experience more precise results are obtained by comparing the minimum doses needed to produce complete protection in a given large proportion of animals, rather than by comparing the doses needed to produce a partial (and generally rather variable) degree of protection. Another source of error is that a slight change in the level at which the cross-section of the tooth is cut greatly affects the picture. The guinea-pig's tooth is of persistent growth and the upper part (away from the root) is that formed before the experimental period, so that if the section be cut high enough it will appear quite normal. More accurate conclusions are formed from longitudinal sections.

2. This value agrees with the amount of ascorbic acid actually present in average specimens of orange juice or in "good" lemon juice, as determined by titration (0.6–0.7 mg. per cc.).

3. Lemon juice is subject to some variation in potency, so that the evaluation of ascorbic acid in terms of International Units will depend on the quality of the lemon juice used for comparison, but taking the interpretation that the standard lemon juice means a "good" specimen, equivalent to the orange juice used in our test and showing 0.6 to 0.7 mg. of ascorbic acid per cc. on titration, ascorbic acid has a potency of 15 International Units per mg. Against "average" lemon juice as purchased retail in Cambridge, having an ascorbic acid content of 0.47 mg. per cc., the calculated activity for ascorbic acid works out rather higher, *viz.* 21 International Units per mg.

4. The alternative value 7.4 units per mg. put forward by Key and Morgan would indicate an ascorbic acid content of 1.35 mg. per cc. for lemon juice, which seems difficult to reconcile with the fact that the reducing titre rarely rises above a value equivalent to about 0.7 mg. Possible reasons for the lack of agreement are discussed.

5. Our earlier conclusion is confirmed, that lemon juice is an unsatisfactory standard and should be replaced by ascorbic acid. Different specimens of lemon juice had ascorbic acid contents varying from 0.19 to 0.69 mg. per cc., average 0.47 with an average deviation of ± 0.11 .

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