CCXLI. THE DETERMINATION OF VITAMIN C BY MEANS OF ITS INFLUENCE ON THE BODY WEIGHT OF GUINEA-PIGS.

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Many workers have found that young guinea-pigs lose weight and die when fed on a diet containing all substances known to be necessary for growth except vitamin C. Other young guinea-pigs fed on the same diet supplemented with vitamin C were found to live and increase in weight. This fact was made the basis for a comparison of the vitamin C content of different samples of apples by Bracewell *et al.* [1930]. Different groups of guinea-pigs were given graded doses of any one sample of apples for periods up to ninety days. The increase in weight and the intensity of the macroscopic lesions indicative of scurvy were used for the assessment of the vitamin C potency of the different doses. The daily doses of apple given to the different groups of animals were generally 3, 5, 10 and 20 g. respectively. The less potent samples gave evidence of their inferiority within four weeks of the beginning of the test. The more potent ones required often as much as eighty or ninety days to produce a difference in response to the different doses. Post mortem examination of the guinea-pigs, either after their natural death during the experimental period, or after they were killed at the end of the experimental period, revealed macroscopic lesions of an intensity which corresponded closely with their decrease or increase in body weight.

Harris & Ray [1932] compared graded doses of fresh ox suprarenal cortex with similarly graded doses of orange juice with respect to their influence both on the structure of the teeth and on the growth of guinea-pigs fed on a scorbutic diet. The respective doses of the two substances (0·25, 0·5, 1·0 and 2·0 g. suprarenal cortex and 0·75, 1·5, 3·0 and 6·0 ml. orange juice) gave practically equal average increases in weight of the groups of animals used and a very smooth curve of response was obtained relating increase in weight of the guinea-pigs to the dose of material given. The curve was very nearly logarithmic in shape for the negative increases in weight, but flattened somewhat for the positive increases. The ratio of the vitamin C potencies of the two substances obtained by this method confirmed the ratio obtained by the "tooth" method.

Thus it appeared that the increase in weight of young guinea-pigs was graded to the dose of vitamin C given, and it seemed to us that a method of estimation of vitamin C based on this property would have distinct advantages over the "tooth" method, provided that it could be carried out within a reasonable length of time, and provided also that it was found to give as great a degree of accuracy as the "tooth" method. It would have the obvious advantage of requiring no histological examination of the teeth with the attendant error involved in the assessment of structural changes, but, on the other hand, it would always be open to the well-known objections to which all tests based on increases in body weight are subject.

In our attempt to work out such a method we obtained a curve of response to graded doses of vitamin C (ascorbic acid, the International Standard of

reference) but the curve, based on average responses of 12 animals in each group, was nearly smooth only after six weeks of feeding. Moreover, the degree of accuracy obtainable in the test, as calculated from the somewhat limited data available, was very similar to or slightly less than that obtainable by the "tooth" method as already worked out in this laboratory. Since our object was to find a simpler and possibly more accurate method than our present one, we did not carry on the experiment for the length of time, 80–90 days, recommended by Zilva. Hence we have no information as to the accuracy of the method as actually used by him. We have, however, concluded that the only advantage of a six weeks' growth test (increase in weight) lies in the avoidance of the histological examination of the teeth, whereas its disadvantages are (*a*) the greater length of time required, (*b*) the possibly lower accuracy and (*c*) the very great one of its not being specific for vitamin C.

If, on the other hand, greater weight is attached to the macroscopic symptoms of scurvy found in autopsies, then the assessment of results is as liable to subjective errors as the assessment in the "tooth" method. In our own experiment we drew up a scheme for assessing the macroscopic symptoms of scurvy found at autopsy and we obtained a curve of response relating the severity of scorbutic symptoms to dose of vitamin C given, which was very similar to the curve found for the increase in weight of the guinea-pigs.

Method.

The guinea-pigs were about 250 g. in weight at the beginning of the experiment. They were not bred in this laboratory, but were obtained from a reliable dealer. They were given large quantities of green food for three days before the experiment began. They were then given a scorbutic diet for the whole of the experiment. The diet consisted of:

Bran	45%
Crushed oats	25%
Dried skimmed milk	30%

A fresh portion of this food was given each day. In addition each guinea-pig was given about 1 ml. of a good sample of cod liver oil twice a week directly into its mouth by pipette, to supply vitamins A and D. Fresh tap water was also given daily. The guinea-pigs were bedded on sawdust, three in each cage (dimensions about 12 in. × 18 in. × 8 in.). They were weighed twice a week.

Different doses of ascorbic acid were given to different groups of animals, each animal of any one group being given the same daily dose for six weeks. At the same time a certain fruit juice was tested also in order to see whether the response to different doses of fruit juice was graded to the dose in the same way (or to the same extent) as the response to different doses of ascorbic acid.

The doses given to the different groups were:

Group 1	No dose
2	0·125 mg. ascorbic acid
3	0·25 mg. ,,
4	0·5 mg. ,,
5	1·0 mg. ,,
6	2·0 mg. ,,
7	0·25 ml. fruit juice
8	0·5 ml. ,,
9	1·0 ml. ,,

The ascorbic acid was given as a solution in glass-distilled water. Fresh solutions were made each day and given to the animals immediately. They were of such concentrations that the required dose was contained in 0·5 or 1·0 ml. water. Each dose was given directly into the back of the guinea-pig's mouth by pipette. The doses were given to different guinea-pigs by four workers at once in order to minimize the time between making the solution and giving it to the guinea-pigs. Dosing was always finished within fifteen minutes of the making of the solutions. The two lower doses of fruit juice were diluted with an equal volume of water and given immediately. In the first part of the experiment only the two lower doses were tested. Each group of guinea-pigs consisted of six animals, but after about three weeks it was evident that the doses of fruit juice were rather low, so twenty-four more guinea-pigs were obtained. Four of these were added to each group receiving the three lowest doses of the Standard, six were added to the group receiving 0·5 ml. fruit juice and six constituted a new group and were given 1·0 ml. fruit juice. It was necessary to divide the fresh batch of guinea-pigs thus, in order to have controls on the Standard if there should prove to be a variation in the whole stock. Actually no such variation was detectable at the end of the experiment, and all the animals were therefore treated as one experiment.

Result.

It had been hoped that a period of four weeks' feeding would be sufficient to bring about a graded response to graded doses of vitamin C, i.e. to produce a reasonably smooth curve of response. When the first six animals in each group had been on experiment for four weeks, however, it was evident that the curve of response obtained by plotting the average increases in weight against the dose of ascorbic acid given was not at all a smooth one. It was therefore decided to make the experimental period six weeks, in the hope of obtaining a smoother curve, but not longer than this, for six weeks was considered the longest period that could be usefully employed in this test, since the method involving the histological examination of the teeth can be completed within four weeks.

The curve of response relating average increase in weight in six weeks to dose of vitamin C (Fig. 1).

The average weights of the groups of animals at weekly intervals during the experiment are given in Table I. The average increases in weight, collected in the last column, were calculated from those guinea-pigs only which survived the six weeks' experimental period. The increases in weight from the different doses of

Table I.

Group	No. of animals	Daily dose of supplement given	Average weight of group of animals at end of week: 0 g.	1 g.	2 g.	3 g.	4 g.	5 g.	6 g.	Average increase in weight of those guinea-pigs (only) which survived six weeks
1	5	No dose	242·8	222·2	228·7 (2 died)	182·3	155·5 (2 died)	145·0 (1 died)	—	—
2	10	0·125 mg. ascorbic acid	245·3	239·2	254·3	248·0	219·1 (2 died)	150·7 (1 died)	143·4 (1 died)	−37·3
3	10	0·25 ,,	243·6	241·4	263·8	277·6	289·3	291·2	276·6	+33·0
4	10	0·5 ,,	242·1	239·9	256·9	268·0	289·6	306·2	304·9	+62·8
5	6	1·0 ,,	250·7	255·2	262·2	276·8	292·2	312·3	321·8	+71·2
6	6	2·0 ,,	239·8	239·0	263·5	267·5	291·0	308·5	324·0	+84·2
7	6	0·25 ml. fruit juice	244·2	239·8	247·5	261·3	220·5	175·5 (3 died)	163·3	−77·7
8	12	0·5 ,,	246·4	246·2	255·0	255·1	234·2	223·0	218·6	−25·5
9	6	1·0 ,,	244·8	245·2	258·5	268·7	274·5	271·5	288·0	+43·2

ascorbic acid were plotted against the doses given, and a curve drawn, by inspection, to connect the points (Fig. 1 A). It was apparently not logarithmic, but nevertheless a curve was drawn, plotting the mean increases in weight against

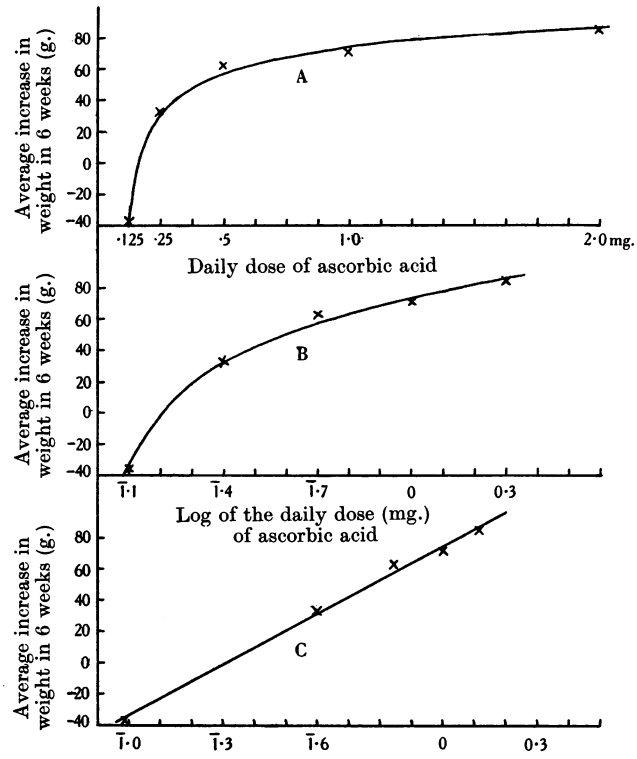

Fig. 1. The mean increases in weight of groups of guinea-pigs given graded doses of ascorbic acid daily for 6 weeks plotted against

(A) the dose of ascorbic acid (x) (equation $y = 74{\cdot}3 + 108{\cdot}2 \log (\log 10x)$);
(B) the log of the dose of ascorbic acid ($\log x$) (equation $y = 74{\cdot}3 + 108{\cdot}2 \log (x + 1)$);
(C) the log of the log of 10 times the dose of ascorbic acid log (log $10x$) (equation $y = 74{\cdot}3 + 108{\cdot}2x$));

to show how the equation of the curve of response was obtained.

the logs of the doses of ascorbic acid. This curve (Fig. 1 B) appeared to be more nearly logarithmic in shape, therefore a fresh curve was drawn relating the mean increase in weight to the log (log dose + 1) (unity was added to get rid of the minus sign of the log of the dose). This proved to be a straight line (Fig. 1 C) whose equation was $y = 74{\cdot}3 + 108{\cdot}2\ x$ where y = the mean increase in weight of the animals and x = log (log dose + 1). Hence the equation relating increase in weight to dose of ascorbic acid given is $y = 74{\cdot}3 + 108{\cdot}2$ log (log $10x$) where y = the increase in weight and x = dose of vitamin C in mg.

Confirmation of the curve of response.

The results obtained by the three doses of fruit juice confirm the shape of the curve of response obtained from the graded doses of ascorbic acid. Since the fruit juice was tested simultaneously with the doses of ascorbic acid which formed

the curve of response, the results from the fruit juice may be compared directly with these results from the ascorbic acid.

Table II.

Daily dose of fruit juice (ml.)	Mean increase in wt. of guinea-pigs (g.)	Abscissa of curve corresponding to increase in wt.	Apparent potency of the fruit juice (mg. ascorbic acid per ml.)	No. of animals used in the test
0·25	−77·7	0·11	0·44	6
0·5	−25·5	0·13	0·26	12
1·0	+43·2	0·33	0·33	6

The result of −77·7 g. increase in weight can only be used if the curve is extrapolated; it is subject to inaccuracy. If this result is omitted, then the weighted mean of the apparent potencies of the juice obtained from the two higher doses, $\frac{12\times0{\cdot}26+6\times0{\cdot}33}{12+6}=0{\cdot}28$, may be taken as the vitamin C potency of the juice (mg. ascorbic acid per ml. juice). If, however, the result from the lowest dose is included, the result becomes 0·32 mg. ascorbic acid per ml. of juice and the potency of the juice may be stated to be, in whole numbers from either figure, 6 I.U. of vitamin C per ml.

Thus the curve of response may be used in future for comparing one dose of the Standard for vitamin C with one dose of the substance under examination in exactly the same way as a curve of response for vitamin A is used.

The accuracy obtainable by this method

The standard deviation of the increase in weight in six weeks of guinea-pigs given doses of vitamin C has been calculated from eight of the groups of animals used in this experiment. As the group given no dose died before the six weeks had ended, it could not be used for the calculation. The method we used was the one used by Coward [1932] in calculating the standard deviation of the results of vitamin A estimations. For the vitamin C tests, the standard deviation of a single determination σ was found to be 32·1. Therefore the probable error (1:1 chance) of the average increase in weight when ten animals are used on one dose is 6·76. The probable error of an estimation depends partly on the probable error of the average increase in weight and partly on the steepness of the curve of response relating increase in weight to dose of vitamin given. The probable error of the increase in weight in this test is high, which would make the probable error of an estimation high, but the curve of response is steep, which would make the probable error of the estimation low. Since the probable error of the mean response of a group of ten guinea-pigs is 6·76, the probable error of the difference between the responses of two groups of guinea-pigs is $6{\cdot}75\times\sqrt{2}=9{\cdot}56$. The probable error of an estimation is then determined by finding the abscissae of the curve of response corresponding to $y=\pm9{\cdot}56$ about the value (mg. ascorbic acid) found by the experiment. For example, suppose a dose of fruit juice had been found to give a response of, say, 50 g. which was approximately equal to that given by a particular dose of the standard. The calculation of the probable error of the result, expressed as a percentage of the value found, is summarized in Table III A.

This is greater than the probable error of an estimation carried out by the "tooth" method when the result is obtained about the middle part of the curve of response.

Since, however, the curve of response in the growth test is not a simple logarithmic one the probable errors will be different for results obtained at different

Table III.

Mean increase in weight (g.)	Abscissa corresponding to the increase in weight	% values	Probable error of result %
	A.		
50	0·394	100	—
50 + 9·56 = 59·56	0·538	136	+36
50 − 9·56 = 40·44	0·306	77	−23
	B.		
0	0·161	100	—
0 + 9·56	0·179	111	+11
0 − 9·56	0·147	91	−9

parts of the curve. Suppose that the dose of fruit juice and the dose of Standard had each been sufficient just to maintain weight in the animals for the six weeks of the test, i.e. the mean increase in weight of the two groups of guinea-pigs had been approximately nil. The probable error of this result is given in Table III B.

The error of a test in which comparison is made with doses which just about produce maintenance in weight of the animals used is therefore relatively low.

Thus this test is more accurate when doses are chosen which just maintain weight in the guinea-pigs than when doses are chosen which bring about a large increase in weight. The dose which will bring about a mean increase in weight of 50 g. is only double the dose which will bring about a mean increase in weight of 10 g. (see Fig. 1 A). Thus there is a very narrow range of effective doses for a vitamin C test. One could not expect often to find that one had chosen the dose that merely produced maintenance of weight, i.e. the one that gave the most accurate result. Hence it is perhaps fairest to state the accuracy of a result obtained by this method as a probable error of +11 to +36 or −9 to −23 %, or an average of +23 and −16 % which is very similar to the error at the middle part of the curve in the "tooth" method.

Thus, it is concluded that the increase in weight of guinea-pigs given abundance of all other substances known to be necessary for growth is dependent on the amount of vitamin C given. This fact may be made the basis for a method of estimation of vitamin C with the procedure, so far found necessary for all biological estimations, of carrying out a simultaneous test of the standard in every estimation made.

Assessment of macroscopic lesions of scurvy.

At autopsy scorbutic symptoms were looked for in the elbow and knee joints (haemorrhages) and in the ribs (swollen costochondral junctions). Different degrees of severity were denoted by the numbers 1–4. The state of each part was assessed separately and the condition of each guinea-pig assessed as the average of the three figures given to the various parts. The average degree of scurvy developed in each group was then calculated. All the animals in group 1 (given no supplement) died within the six weeks of the test and most of them had developed only slight signs of scurvy. The other groups developed scurvy to an extent proportional to their deprivation of vitamin C in a curvilinear relationship. The results are collected in Table IV.

A calculation of the relationship between the average intensity of scurvy developed in six weeks and the daily dose of vitamin C given was made in the same way as the calculation relating average increase in weight and dose of vitamin C given (Fig. 2 A, B, C). The relationship was found to be expressed by

Table IV.

Group	Number of animals	Daily dose of supplement given	Average degree of scurvy developed
1	5	No dose	1·0
2	10	0·125 mg. ascorbic acid	1·83
3	10	0·25 ,,	1·55
4	10	0·5 ,,	0·67
5	6	1·0 ,,	0·33
6	6	2·0 ,,	0·25
7	6	0·25 ml. fruit juice	2·54
8	12	0·5 ,,	1·59
9	6	1·0	0·75

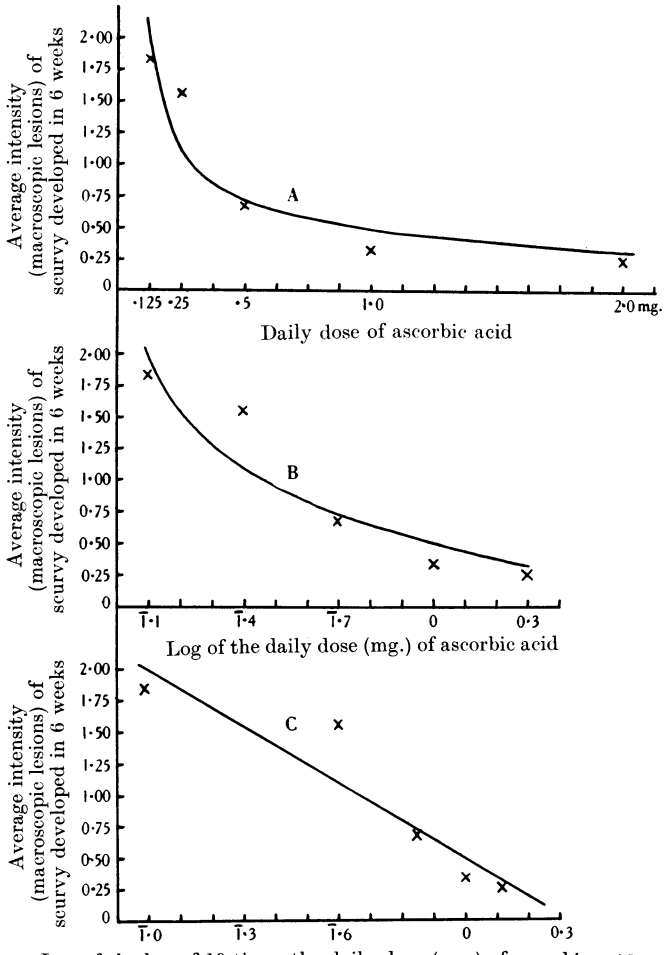

Fig. 2. The average intensity of scurvy developed in groups of guinea-pigs given graded doses of ascorbic acid daily for 6 weeks plotted against

(A) the dose of ascorbic acid (x) (equation $y = 0{\cdot}49 - 1{\cdot}50 \log (\log 10x)$);
(B) the log of the dose of ascorbic acid log (x) (equation $y = 0{\cdot}49 - 1{\cdot}50 \log (x+1)$);
(C) the log of the log of 10 times the dose of ascorbic acid log (log $10x$) (equation $y = 0{\cdot}49 - 1{\cdot}50x$);

to show how the equation of the curve of response was obtained.

the equation $y=0{\cdot}49-1{\cdot}50$ log (log $10x$), where $y=$the intensity of scurvy developed (as measured by the particular scheme adopted) and $x=$the daily dose (in mg.) of ascorbic acid given. How nearly the points fit this curve may be seen in Fig. 2 A. It is evident that four of the five points fit a curve slightly below this one remarkably well but the fifth point raises the whole curve.

By substituting in the equation $y=0{\cdot}49-1{\cdot}50$ log (log $10x$) the values of y obtained by giving the three different doses of fruit juice, the following values were found:

0·25 ml. fruit juice contained 0·110 mg. ascorbic acid.
0·5 ml. fruit juice contained 0·164 mg. ascorbic acid.
1·0 ml. fruit juice contained 0·469 mg. ascorbic acid.

These three results give the potency of the juice as 0·44, 0·33 and 0·47 mg. respectively of ascorbic acid per ml. The average, weighted according to the number of animals in each group, is 0·39 mg. ascorbic acid per ml. of juice. Therefore the juice contains about 8 I.U. of vitamin C per ml.

A simpler calculation for the potency may be made in the following way.

(*a*) 1 ml. fruit juice gave slightly less protection than 0·5 mg. ascorbic acid.

Therefore 1 ml. fruit juice appears to contain about 8 I.U. of vitamin C.

(*b*) 0·5 ml. fruit juice gave the same amount of protection as 0·25 mg. ascorbic acid.

Therefore 1·0 ml. fruit juice appears to contain 10 I.U. of vitamin C.

(*c*) 0·25 ml. fruit juice gave less protection than 0·125 mg. ascorbic acid.

Therefore 1·0 ml. fruit juice appears to contain about 7 I.U. of vitamin C.

The average of the three results (*a*), (*b*) and (*c*) is about 8 I.U. of vitamin C per ml. of juice, which is the same result as that obtained by the more elaborate calculation and may be considered a fair confirmation of the value, 6 I.U. of vitamin C per ml. of juice, found by the method based on increase in weight. Therefore, the estimation of the vitamin C potency of a substance obtained by comparing the average intensity of scurvy developed in the animals given doses of that substance with that in animals given doses of the International Standard at the same time, gives the same result as a comparison of the increases in weight of the same animals during the experiment.

The amount of ascorbic acid present in the fruit juice was not determined by the chemical method as sulphur dioxide had been used for its preservation.

Comparison of this method with the "tooth" method.

The method of estimating vitamin C by its influence on the body weight of guinea-pigs during a period of six weeks is no more accurate than the method of estimating it by its influence on the histological structure of the teeth during a period of two weeks. It has two advantages over the "tooth" method, (*a*) it is independent of the subjective errors of assessment of the amount of scurvy developed, (*b*) it does not involve the rather skilled technique of cutting the sections of the teeth. On the other hand, the "tooth" method of estimation can be completed within a period of four weeks and also it has the very great recommendation of being based on a reaction specific for vitamin C. It seems to us, therefore, that the "tooth" method is still very much to be preferred to the "increase in weight" method as worked out by us.

SUMMARY.

A method for the estimation of vitamin C has been worked out on the basis of its influence on the body weight of guinea-pigs. The method is very similar to the method of estimating vitamin A which was worked out in this laboratory.

Different groups of guinea-pigs were given daily doses of 0·125, 0·25, 0·5, 1·0 and 2·0 mg. of ascorbic acid (International Standard), each guinea-pig in any one group being given the same daily dose. A diet containing abundance of all other substances known to be necessary for growth was given *ad lib.*, a fresh portion each day. The guinea-pigs were weighed twice a week. When the test had been carried on for six weeks a fairly smooth curve of response was obtained relating increase in weight to dose of vitamin C given. In any period shorter than six weeks, the curve of response was much less smooth. The curve of response was represented by the equation $y=74{\cdot}3+108{\cdot}2 \log (\log 10x)$.

The severity of the scurvy developed by the animals during the test also bore a curvilinear relationship to the dose of vitamin C given. A fairly good curve of response, $y=0{\cdot}49-1{\cdot}50 \log (\log 10x)$, was obtained.

A sample of fruit juice examined by a test carried out simultaneously with the tests on the ascorbic acid (International Standard) was found to contain 6 I.U. of vitamin C per ml. as estimated by the increase in weight of the animals, and 8 I.U. per ml. as estimated by the severity of scurvy developed. This was considered to be fairly good agreement.

The accuracy of the "increase in weight" method has been calculated. It is not greater than the accuracy of the "tooth" method.

The relative merits of the two methods have been discussed. It is concluded that the "tooth" method has distinct advantages over the "increase in weight" method as carried out in the experiment described in this paper.

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