C. A QUANTITATIVE METHOD FOR THE DETERMINATION OF VITAMIN C.

BY KATHLEEN MARY KEY AND GORDON KIRBY ELPHICK.

From the Pharmacological Laboratories of the Pharmaceutical Society of Great Britain.

(Received May 1st, 1931.)

It has been shown by Zilva and Wells [1919], Höjer [1924, 1926], Goettsch [1928] and others that a scorbutic diet will produce histological changes in the teeth of young guinea-pigs. A transverse section of the root of the incisor of a young, normal guinea-pig shows a layer of long parallel odontoblasts surrounding the pulp and separated from the regular dentine by a narrow predentine. Fine canals cross the predentine and dentine from the odontoblasts. In a scorbutic tooth the odontoblast layer is disorganised, the predentine is calcified and an irregular layer of bone is formed between the predentine and the odontoblasts. The dentine canals are fewer and are only found radiating from the predentine outwards. Höjer described ten stages in the development of scurvy based upon the amount of disorganisation of the odontoblasts, the width and irregularity of the inner dentine or layer of bone, the degree of calcification of the predentine and the structure of the dentine. He expressed the amount of protection from the disease afforded by any dose, as a fraction of complete protection, and he claimed that this fraction represented the ratio of the test dose to the minimum dose which would give complete protection. Goettsch [1928] repeated Höjer's method and found that the great individual variations of the animals made it difficult to determine the fractions of the minimum protective dose. She could, however, find a dose such that all the animals receiving it were fully protected from scurvy and those receiving lower doses were partially protected in varying degrees. She compared the potency of two substances by determining the minimum dose of each which would produce complete protection in all the guinea-pigs given that dose. The ratio of the potency of the two substances was given by the inverse ratio of the minimum protective doses. Goettsch also determined the minimum protective dose of a substance by Zilva's method in which the diagnosis of scurvy is based on growth, haemorrhages of the joints, swelling and histological appearance of the rib junctions. She found that the minimum protective dose determined by Zilva's method was approximately one-half the dose necessary in Höjer's test. The ratio of the minimum protective doses of two substances was, however, the same in both tests. In these experiments no use could be made of results given

by animals receiving doses which were less than the minimum fully protective dose. If, as appears probable from the work of Goettsch, it may be assumed that using her diet the appearance of the teeth is entirely dependent upon the amount of vitamin C present, then it follows that the degree of scurvy produced must be graded to the dose of vitamin C, provided that sufficient animals are given each dose to eliminate differences due to individual variations. If a relation could be found between the average amount of protection given by a dose and the dose, then the dose which would produce full protection could be calculated for an unknown substance. The method used by us for determining the antiscorbutic potency of graded doses of orange juice was based on the results of Höjer and Goettsch.

The oranges were bought in London and stored at a temperature of about 5°. A fresh orange was cut each day and the juice was expressed and strained through muslin before being given to the animals through a graduated pipette. The guinea-pigs were imported one dozen at a time and were kept in the laboratory for 24 hours before the beginning of each test. During that period they were given a complete diet of oats, bran and plenty of greenstuff. The first batch of oranges was bought on November 7th, 1930, and used for feeding three dozen guinea-pigs which were received in the laboratory on November 6th, 1930, December 9th, 1930, and January 1st, 1931, respectively. The second batch of oranges was bought on January 23rd, 1931, and used for feeding two dozen guinea-pigs received on January 22nd and February 12th respectively.

All the guinea-pigs used for the test were given unlimited quantities of a basal diet consisting of bran 45 %, crushed oats 25 %, and dried skimmed milk 30 %, together with 1 cc. cod-liver oil twice weekly and tap-water *ad lib.*

Each set of one dozen guinea-pigs was divided into four groups of three animals and the members of each group received no dose, and daily doses of 0.75 cc., 1.5 cc., 3.0 cc. orange juice respectively. The weight of each animal was recorded twice weekly. The test was continued for 14 days, then all the animals were killed and examined for any macroscopic lesions. Their jaw-bones were placed in Jenkin's decalcifying solution. The growth response, *post mortem* observations and structure of the roots of the incisors are detailed below.

(a) *Growth responses.*

Guinea-pigs weighing between 250 g. and 350 g. were chosen for the test. The average initial weight was 293 g. In Fig. 1 composite growth curves are shown for the animals on each dose. Good growth was obtained in each group and there was no appreciable difference between the growth of the animals receiving only the basal diet and those receiving orange juice in addition. There is no evidence to show that the slower rate of growth of the control animals during the last few days of the test was in any way due to the onset of scurvy. Great variation was found in the growth of the individuals in each group, as will be seen from Table I.

Table I.

Daily dose of orange juice cc.	Greatest increase in weight of any individual in group g.	Least increase in weight of any individual in group g.	Average increase in weight g.
No dose	75	5	38
0.75	85	- 5	50
1.5	75	20	46
3.0	80	15	48

These results show that, for a test of such short duration as 14 days, the growth of a guinea-pig cannot be taken as any indication of the antiscorbutic value of its diet.

Fig. 1. Composite growth curves (average weights of 15 animals in each group).

(b) *Post mortem examination.*

When they were killed all the 59 animals used in the experiment appeared to be in good, healthy condition except seven which had abscesses on the neck. Five others were found to have small neck abscesses when *post mortem* examinations were made. Special care was taken to note any haemorrhages at the knees or ribs and any swelling of the costochondral junctions of the ribs. These effects were, however, slight compared with those obtained in animals suffering from severe scurvy due to prolonged feeding on a deficient diet. The results are shown in Table II.

Table II.

Daily dose of orange juice cc.	No. of animals showing any haemorrhages at joints	No. of animals showing swelling of rib junctions	No. of animals with abscesses on neck	Remarks
No dose	11	2	5	3 animals had slight congestion of lungs.
0.75	6	.	2	1 animal had slight congestion of lungs. 2 animals had slight infection in stomach and intestines.
1.5	3	1	3	1 animal had slight congestion of lungs.
3.0	5	.	2	2 animals had slight congestion of lungs.

Degree of protection from scurvy 0

Degree of protection from scurvy 1

Degree of protection from scurvy 2

Degree of protection from scurvy 3

Degree of protection from scurvy 4

It will be seen that 11 of the 15 animals receiving no orange juice developed some haemorrhages at the joints. In the other groups the proportion of affected animals was lower. Similarly, two animals amongst those receiving no orange juice showed swelling of the rib junctions and five had abscesses on the neck. The corresponding figures for the other groups were lower. Thus macroscopic *post mortem* examinations showed that at the end of 14 days some difference could be detected between a scorbutic diet and a similar diet supplemented with orange juice, but no distinction could be made between the effect of different amounts of orange juice.

(c) *Structure of transverse sections of incisor roots.*

The method finally used for determining the relative potency of different doses of orange juice was based solely on the histological appearance of the roots of the incisors. In order to obtain a numerical value for the degree of scurvy produced in each animal, an arbitrary scale was devised in which the figures 0 to 4 represented stages from severe scurvy to complete protection from the disease. Plates IV and V show the characteristic appearance of transverse sections of the incisors in each group. A detailed description of the groups is given below and a summary will be found in Table III.

Group 0. In this group the odontoblasts were completely or almost completely disorganised. The inner dentine was wide, being approximately one-third the width of the dentine itself. It projected into the pulp and in some sections completely enclosed small portions of the pulp. The predentine was amorphously calcified and the Tomes's canals occurred only in the outer dentine. This picture represented the severest form of scurvy which we had produced in 14 days.

Group 1. The odontoblasts in this group were completely disorganised in some parts of the section, but in other places they were short and parallel, being only disorganised near the pulp. The inner dentine was narrower than in group 0 and although it was irregular near the odontoblasts, there were no long projections into the pulp. The predentine was calcified. In some of the animals included in this group the Tomes's canals occurred only in the outer dentine, in others a few canals could also be seen in the inner dentine.

Group 2. This group could be distinguished from the preceding ones in that the odontoblasts were all parallel though short and disorganised near the pulp. The inner dentine was narrow and regular, the predentine was either calcified or partially calcified and some Tomes's canals occurred in the inner dentine.

Group 3. The animals included in this group had only slight scurvy. The first part of the tooth to become affected by any lack of vitamin C in the diet appears to be the odontoblast layer. The cells, which are normally long and parallel, become disintegrated towards the pulp though still retaining their long and parallel formation. Teeth showing odontoblasts of this type were included in group 3 when there was no inner dentine and the predentine and Tomes's canals were normal. A few animals had teeth which were normal in

all respects except for a mere rim of inner dentine and slight disintegration of the odontoblasts. These were also included in this group.

Group 4. The animals in this group were completely protected from the onset of scurvy and consequently their teeth were normal. The odontoblasts were long and parallel. There was no inner dentine. The predentine was not calcified and the Tomes's canals crossed the dentine from the odontoblasts.

It was found that nearly all the teeth examined would fit into one of these five groups. There were a few exceptional animals in which one part of the tooth would indicate severe scurvy and justify inclusion in group 0, while another part of the section conformed with group 3 or 4. For example, a wide inner dentine was sometimes found accompanied by long, parallel odontoblasts, only slightly disorganised near the pulp. Such sections were judged separately by two individual workers and an average value for the degree of protection from scurvy was determined. In two teeth a series of two or three calcified rings could be seen in the dentine, resembling a series of calcified predentines. We could not understand the origin and interpretation of this structure.

In a normal tooth the dentine is often very wide, even approximately equal in thickness to the widest part of the pulp. In a scorbutic tooth the dentine is comparatively narrow, but since the width depends to some extent upon the exact position of the transverse section, this feature cannot be used as a criterion for determining the degree of scurvy.

Each guinea-pig used in the test was assigned a value for the degree of protection from scurvy in accordance with the scheme which is summarised in Table III. The results are shown in Table IV.

Table III.

Odontoblasts	Inner dentine	Predentine	Tomes's canals	Degree of protection from scurvy
Disorganised	Wide, with projections into pulp	Calcified	Only in outer dentine	0
Completely disorganised in places but some parallel formation	Narrow and irregular	Calcified	Only in outer dentine or mostly in outer dentine	1
All parallel but becoming disorganised near pulp	Narrow	Calcified or partially calcified	Mostly in outer dentine or crossing inner and outer dentine from odontoblasts	2
Long and parallel but becoming disorganised near pulp	Absent or mere rim	Not calcified	Cross dentine from odontoblasts	3
Long and parallel	Absent	Not calcified	Cross dentine from odontoblasts	4

The teeth of all the animals which received no orange juice showed severe or moderately severe scurvy and the degree of protection was estimated as

Table IV.

No dose		0.75 cc. orange juice		1.5 cc. orange juice		3.0 cc. orange juice	
Guinea-pig	Degree of protection from scurvy	Guinea-pig	Degree of protection from scurvy	Guinea-pig	Degree of protection from scurvy	Guinea-pig	Degree of protection from scurvy
1	0	4	0	7	3	10	Died on 4th day of test
2	0	5	2	8	1	11	4
3	0	6	1	9	0	12	3
13	0	16	2	19	3	22	4
14	0	17	2	20	3	23	4
15	0	18	2	21	3	24	4
25	1	28	0	31	2	34	3.5
26	1	29	1	32	3	35	3.5
27	1	30	1	33	2	36	4
37	1	40	1	43	3	46	4
38	1	41	2	44	4	47	4
39	1	42	1	45	2	48	4
49	1	52	2.5	55	3	58	4
50	1	53	3	56	1	59	4
51	1	54	1	57	3	60	4
Average value	0.6		1.4		2.4		3.9

0 or 1 according to the scheme in Table III. 3.0 cc. orange juice was the highest dose tested and this produced complete protection in 11 of the 14 animals used, and nearly complete protection in the remaining three. The effect of the lower doses of orange juice was more irregular. For example, among the 15 guinea-pigs given a daily dose of 1.5 cc. orange juice, one was assigned the value 0 and one the value 4, the remaining animals being given intermediate figures. It was assumed that the average value for the 15 animals receiving each dose represented the true protective power for that dose as measured by our arbitrary scheme. The average value for the degree of protection from scurvy was plotted against the dose of orange juice and a straight line was obtained as shown in Fig. 2.

Fig. 2.

This curve could be used to compare any unknown substance with any standard. Suppose, for example, the potency of the unknown were required in

terms of decitrated lemon juice¹. Some guinea-pigs would be given the unknown and others the lemon juice. The average degree of protection given by each dose of unknown and by each dose of lemon juice would be determined. Suppose the average degree of protection given by 1.5 cc. lemon juice were 2.4 and that given by a similar dose of unknown, 1.6, then the relation between the lemon juice and the unknown would be given by the ratio of the corresponding abscissae obtained from Fig. 2. Thus

$$\frac{\text{Potency of unknown}}{\text{Potency of lemon juice}} = \frac{0.85}{1.5} = 0.6.$$

Orange juice compared with lemon juice as standard.

Orange juice is useful as a standard of reference for vitamin C since it is easily obtained and can be given directly to the animals without previous treatment. The guinea-pigs like the juice and drink it readily. Lemon juice is unpalatable unless decitrated and the process of decitration is difficult to control on the small scale. If insufficient calcium carbonate is added, a cloudy filtrate is obtained even after standing for two or three hours. If too much calcium carbonate is added, very little juice is obtained. Different lemons require different amounts of calcium carbonate for the best yield of decitrated juice. It is a disadvantage that a standard should require preliminary treatment of this sort, which differs for different samples of lemon juice. Orange juice, is, therefore, more convenient as a standard if it may be assumed that its potency is equally constant. In our experiments no difference in activity has been detected between the fresh oranges and those which had been stored for three months at 5°. Also there appeared to be no seasonal variation in the response of the guinea-pigs during the four months November to February. These results were, however, inconclusive and require further investigation. During the period from October 1928 to August 1930, 28 guinea-pigs were given a daily dose of 3 cc. orange juice. They were included in eight different experiments at different times of the year. All the animals were fully protected from scurvy except one which showed slight symptoms of the disease. It appears from these results that the antiscorbutic potency of orange juice is probably just as constant for different oranges as is the antiscorbutic potency of lemon juice.

Determination of the antiscorbutic potency of an unknown substance in terms of the potency of orange juice.

The dose of orange juice whose antiscorbutic activity is equal to that of a given dose of unknown may be determined from Fig. 2. The average value for the degree of protection from scurvy given by the unknown is found by experiment and the corresponding dose of orange juice is obtained directly from the curve.

¹ The use of lemon or orange juice as a standard of antiscorbutic potency has been provisionally recommended by a Sub-committee of the Committee on Accessory Food Factors appointed by the Medical Research Council and Lister Institute.

Exp. 1. In a test of Bramley's seedling apples in which three animals were used for each dose the following results were obtained:

Daily dose of apple = A g.	Degree of protection from scurvy shown by each animal on dose	Average degree of protection for each dose	Corresponding dose of orange juice = B (From Fig. 2) cc.	Dose of orange juice equivalent to 1 g. apple = B/A cc.
1.5	1 0 1	0.67	—	—
3.0	1 0.5 2	1.17	0.5	0.17
6.0	4 3 0	2.33	1.47	0.24

Thus the degrees of protection produced in the three animals receiving 3 g. apple were 1, 0.5 and 2 respectively. The average of these is 1.17. From the curve in Fig. 2 it will be seen that the abscissa corresponding with 1.17 degrees of protection gives 0.5 cc. as the equivalent dose of orange juice. The vitamin C content of 3 g. apple is, therefore, equal to that of 0.5 cc. orange juice and the dose of orange juice equal to 1 g. apple is 0.5/3 or 0.17 cc. Similarly 6 g. apple were found to be equivalent to 1.4 cc. orange juice, therefore, 1 g. apple is equivalent to 0.24 cc. orange juice.

Thus the antiscorbutic potency of 1 g. apple is approximately equal to that of 0.2 cc. orange juice. Greater accuracy could be obtained by the use of more animals on each dose. The error caused by individual variations must be very great when one animal out of the three receiving 6 g. apple gave the value 0 and another gave the value 4. In the following experiment five animals were used for each dose.

Exp. II. A sample of diluted tomato juice of unknown strength was examined for its vitamin C content. Daily doses of 3 cc., 6 cc., and 12 cc. were chosen and five guinea-pigs were used for each dose. The results were estimated independently by two experienced and two inexperienced observers. The results are shown in Table V.

Table V.

Dose of diluted tomato juice = A cc.	No. of guinea-pig	Values assigned by				Average for each animal	Average for each dose	Corresponding dose of orange juice (from Fig. 2) = B cc.	Dose of orange juice equivalent to 1 cc. tomato juice = B/A cc.
		KK	GE	FD*	BM*				
3	61	4	4	4	4	4.0	1.4	0.7	0.23
	62	2	1.5	2	1	1.6			
	63	0.5	0	1	1	0.6			
	64	0	0	0.5	0.5	0.2			
	65	0.5	0	1	0	0.4			
6	66	3	3	3	2	2.7	2.2	1.4	0.23
	67	0	0	0	0	0			
	68	4	4	4	3	3.7			
	69	3	3	3	2	2.7			
	70	2	1.5	2	2	1.9			
12	71	4	4	4	4	4.0	3.7	2.7	0.225
	72	4	4	3	4	3.7			
	73	3	3	3	3	3.0			
	74	4	4	3.5	4	3.9			
	75	4	4	3.5	3.5	3.7			

* Observers with very little experience of normal and scorbutic teeth. Their estimates were given entirely from Plates IV and V and Table III.

It will be seen that the independent observations were in close agreement and that the three doses gave the same value for the potency of the tomato juice. This result is probably less accurate than the figures appear to indicate but the experiment shows that when five animals are given each dose, the differences due to individual variations are to a large extent eliminated.

DISCUSSION.

The method described in this paper shows that graded responses can be obtained with different doses of vitamin C when the average protective value is determined for each dose. The curve relating the response to the dose may be used for determining the potency of an unknown substance in terms of any standard. This result is more accurate than that obtained from the minimum protective doses as recommended by Goettsch. Suppose, for example, an unknown fruit juice were examined for its vitamin C content. In order to determine the minimum protective dose, it would be necessary to test a wide range of doses, 0.5 cc., 1 cc., 2 cc., 4 cc. and 8 cc. might be suitable daily doses. Suppose three animals were used for each dose and all those receiving 4 cc. and 8 cc. were fully protected from scurvy while those receiving 2 cc. were not fully protected. Then the minimum protective dose of the unknown is less than 4 cc. but more than 2 cc. Since the minimum protective dose of orange juice is 3 cc., the ratio

$\frac{\text{Potency of unknown}}{\text{Potency of orange juice}}$ is greater than $\frac{2}{3}$ or 0.75 and less than $\frac{3}{2}$ or 1.5.

If the same fruit juice were examined by the method which is here described, fewer doses would be required though a wide range would still be necessary in order to obtain at least one dose which would give partial protection. Suppose the fifteen animals were divided into three groups of five individuals and the doses chosen for each group were 0.5 cc., 2 cc., and 8 cc. respectively. The following results might be obtained:

Daily dose of unknown = A cc.	Average degree of protection	Corresponding dose of orange juice = B (From Fig. 2) cc.	Dose of orange juice equivalent to 1 cc. of unknown = B/A cc.
0.5	1.3	0.6	1.2
2.0	2.9	2.0	1.0
8.0	4.0	Not < 3.0	Not < 0.4

Obviously 8 cc. is more than enough to produce complete protection. It is therefore more potent than 3 cc. orange juice. No further information can be gained from this dose. The lower doses show that the ratio

$\frac{\text{Potency of unknown}}{\text{Potency of orange juice}}$ equals approximately 1.1

since this is the average of the values in the right-hand column. This result agrees with that obtained by the minimum protective dose method but gives a more precise result.

Similar results could be obtained with decitrated lemon juice or any other standard.

This method is particularly useful for determining the antiscorbutic potency of substances containing little vitamin C. It is often difficult to give guinea-pigs high enough doses of such substances to produce complete protection from scurvy so that the minimum protective dose cannot be directly determined. Also when testing an unknown substance, a dose might be chosen which would produce partial but not complete protection. The corresponding dose of orange juice could be directly determined from the curve in Fig. 2.

SUMMARY.

A method is described for determining the antiscorbutic potency of a substance in terms of the potency of a standard such as lemon juice or orange juice. Different doses of orange juice were examined on a series of guinea-pigs and the amount of protection from scurvy produced in each animal was determined by means of an arbitrary scale. A curve was constructed relating the average amount of protection afforded to the dose of orange juice given. A straight line was obtained and this was used as a curve of reference for evaluating results on an unknown substance. Some disadvantages of the use of decitrated lemon juice as a standard are pointed out, and evidence is given which suggests that the antiscorbutic potency of orange juice is constant.

REFERENCES.

- Goettsch (1928). *Quart. J. Pharm.* 1, 168.
Höjer (1924). *Acta Pediat.* 3 (suppl.).
— (1926). *Brit. J. Exp. Path.* 7, 356.
Zilva and Wells (1919). *Proc. Roy. Soc. Lond.* B 90, 505.

DESCRIPTION OF PLATES IV AND V.

PLATE IV. *d.* dentine showing Tomes's canals. *p.* calcified predentine. *i.d.* inner dentine. *o.* odontoblasts. *pu.* pulp.

PLATE V. *d.* dentine showing Tomes's canals. *p.* uncalcified predentine showing Tomes's canals. *i.d.* inner dentine. *o.* odontoblasts. *pu.* pulp.