THE GROWTH OF THE ODONTOBLASTS OF THE INCISOR TOOTH AS A CRITERION OF THE VITAMIN C INTAKE OF THE GUINEA PIG [1]

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FIVE FIGURES

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After describing briefly 2 physical methods, 21 chemical methods, and 1 biochemical method, Rosenberg ('45) stated "Although the chemical, and to a small extent also, physical methods are replacing more and more the biological determinations of vitamin C, the biological tests maintain their place as the ultimate and most correct method of determining vitamin C." The problem of the assay of this vitamin was of particular concern to the Canadian Government during the war years because of the difficulty of providing natural sources of vitamin C to the armed forces for a considerable portion of the year. Inasmuch as different chemical procedures frequently gave different results as to the potency of a food in which the armed forces were interested, this laboratory was requested in 1942 to undertake the establishment of a vitamin C bioassay which might be used as a check against chemical procedures.

THE BASAL DIET

Most biological assays for vitamins depend ultimately on the normal development of the experimental animal, either as

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a whole, as measured by growth; or of some tissue which can be observed separately and on which the vitamin appears to have specific effects. Since the development of the tissues of the body may be limited through diet inadequacies, it is obviously essential that any diet, to be fully satisfactory as the basis of a bioassay, must be nutritionally complete with the exception of the vitamin to be studied.

Insofar as we are aware, the nutritional requirements of the guinea pig are not as yet completely known — in the sense at least that it has not yet been possible to provide either a purified diet or one of natural foods which is fully successful in maintaining normal growth and reproduction, unless some fresh herbage is also fed. Several of the diets, fortified with ascorbic acid, which have been reported for the bioassay of vitamin C have proved in this laboratory to be entirely inadequate to support a successful pregnancy or to promote normal growth of young pigs to maturity.

We were faced at once, therefore, with the necessity of developing a basal diet for the assay. Suitable criteria of nutritional adequacy offered some problems, but due to the fact that the young guinea pig has a particularly long intrauterine life, and that it is physiologically much more mature at birth than most other small animals, it was believed that dietary deficiencies might be manifest by reproductive failure or by sub-normal performance of breeding females confined to such rations. It was decided, therefore, to feed to pregnant guinea pigs, different basal diets suitable with respect to such factors as palatability, physical nature and freedom from vitamin C, and to note their ability to complete normal reproduction. Comparable females, fed the same diets plus green feed, were used as controls. All test basal diets were supplemented with ascorbic acid as the sole source of vitamin C.

In addition to the pregnancy tests, the more promising of the mixtures were subjected to growth-tests with groups of young animals fed against check groups receiving the same rations plus green feed.

As a result of these studies a basal diet has been devised for the vitamin C bioassay. It is called the Macdonald Guinea Pig Basal Diet no. 5, and has the following percentage composition: ground oats 15, ground wheat 13,[2] ground, dried beet-pulp 25, linseed oilmeal 12.5, skimmilk powder 15, fishmeal 5, brewer's dried yeast 10, bone char 4, and salt (iodized) 0.5.

For feeding, the mixture is pressed into pellets of about one-eighth inch diameter. We have found that while guinea pigs will scratch a meal mixture out of their feeders, they will eat the same mixture without waste when it is offered in small pellet form. In addition to the dry basal mixture, the pigs were supplied directly with: vitamins A and D as a feeding fish oil; vitamin E as alpha tocopherol; and vitamin C as ascorbic acid. It may be noted that wheat germ to the extent of 10% of the basal mixture did not prevent muscle degeneration and hemorrhage which were entirely corrected by daily allowances of 3 mg of alpha tocopherol.

In our experience, when this vitamin-supplemented ration is fed ad libitum along with either fresh or with dried, long-stored grass clippings, reproduction (80% successful pregnancies) and the growth of young are normal; whereas if the roughage is omitted, only about 66% of pregnancies are successful and there is some, though not a marked slowing of growth of the young to maturity. It may be noted that the dried herbage clippings used were not anti-scorbutic. Because of the evident craving for edible roughage, it is interesting to speculate as to whether or not some of the failure which has been reported in the preparation of purified diets for guinea pigs is not related to the need of this species for roughage material to maintain normal caecum function, comparable to the case of the rumen with ruminants.

From our observations of several hundred pregnant guinea pigs and of their progeny, we have now constructed what may

[2] Five pounds of this component may be replaced, if desired by an equal weight of molasses for greater ease in pelleting.

be called a "normal standard" for the behavior of guinea pigs on this diet regime. It may be summarized as in table 1.

Although it is evident from these tests that the diet which we are using without roughage for the odontoblast assay is not entirely complete nutritionally when judged by the severe criterion of adequacy for reproduction, yet young pigs carried on bioassay, on this diet, show no abnormalities at post-mortem examination at the end of the 42-day assay period provided the ascorbic acid has been supplied in amounts of 2 mg per pig per day or greater.

TABLE 1

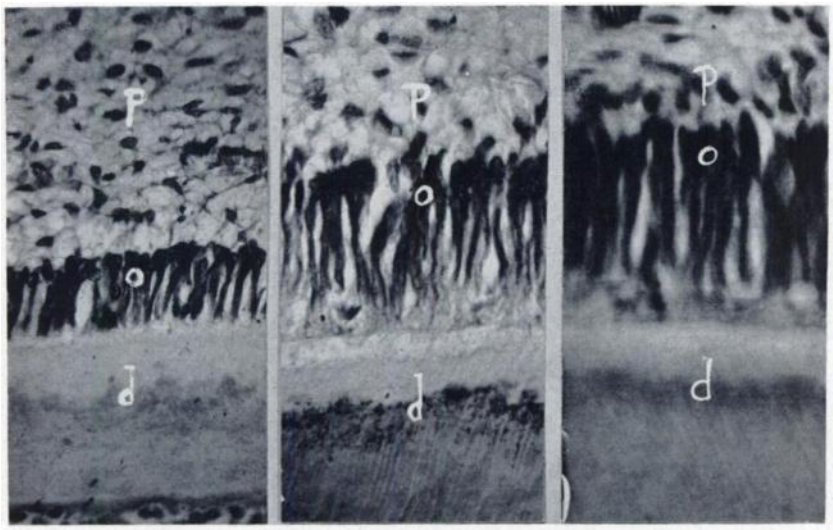
Typical average performance of guinea pigs on vitamins A, C, D and E supplemented Macdonald guinea pig diet no. 5.

RESPONSE WITH REGARD TO:	WITHOUT ROUGHAGE	WITH FRESH OR DRIED ROUGHAGE
Successful pregnancies	67%	80%
Hemorrhage and resorption of fetus	20%	20%
Abortion	13%	0%
Number of pigs per litter:		
total	3.7	3.9
born alive	3.0	3.5
weaned	3.0	3.5
Birth weight of pigs born alive	100 gm	100 gm
Weight at 21 days (weaning time)	260 gm	275 gm
Gains per wk. from weaning time to 500 gm (females only)	30 gm	43 gm

The bioassay procedure

Numerous workers have described the radical changes which occur in the scorbutic guinea pig tooth and these changes have been used in subjective assay methods as an index of the vitamin C intake of young guinea pigs. All of these methods suffer from an inherent lack of precision. In 1940, an objective method of assay was proposed by Boyle, Bessey and Howe based on their finding that the width of the dentine layer of the incisor tooth varied with the vitamin C intake. This method

appeared to avoid the difficulty of assessing the degree of tooth damage involved in earlier tooth assay methods. However, in examining the teeth of pigs on varying intakes of ascorbic acid, we were impressed with the clear-cut and easily measured differences in the length of the odontoblast cells (see fig. 1).



0.5 mg 1.0 mg 2.0 mg

Fig. 1 Photomicrographs (× 440) showing development of the odontoblast cells in guinea pig incisor teeth resulting from the daily intake of 0.5, 1.0, and 2.0 mg of ascorbic acid, respectively. P, o, and d indicate pulp, odontoblasts and dentine, respectively.

In the mildly scorbutic guinea pigs the odontoblast cells at the formative end of the incisor tooth appear practically normal, while further incisally they become shorter and irregular in position (see fig. 2).

In the normal guinea pig, the odontoblasts, differentiating at the formative end of the tooth, increase in length until they reach maturity. At the senile end of the tooth, the odontoblasts have degenerated and are embedded in calcific scar tissue, or secondary dentine. Between these 2 well-defined

regions lies a row of tall columnar mature odontoblasts. Cell measurements must be taken in this area (figs. 3, 4 and 5).

These changes can be seen in longitudinal sections cut to expose the full length of the pulp cavity of the incisor tooth

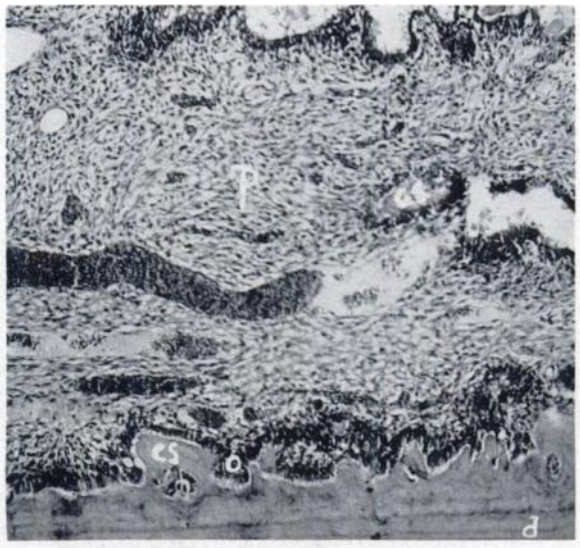


Fig. 2 Photomicrograph (× 100) showing irregular odontoblast row in a scorbutic tooth. (P) indicates disorganized pulp, (cs) calcific scar tissue, (o) degenerating odontoblasts, and (d) dentine, respectively.

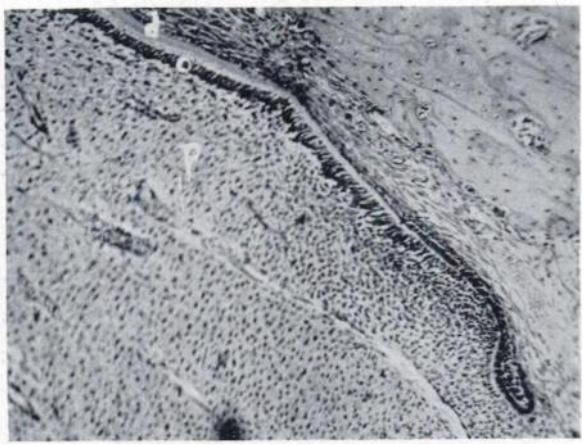


Fig. 3 Formative end of incisor tooth showing embryonic odontoblasts.

and are easily measured with a microscope fitted with a micrometer eye piece.

The length to which these cells develop in pigs of 250 to 400 gm weight, is apparently limited by the level of vitamin C intake, and ranges from about 30 microns with intakes of 0.5 mg of ascorbic acid daily, to a maximum of about 70 microns with intakes of 2 mg or over. Within this range of

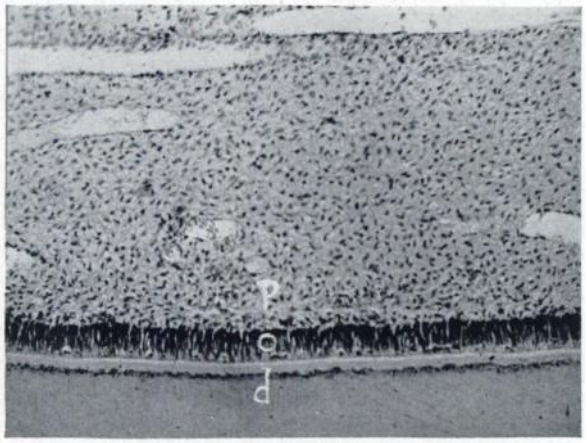


Fig. 4 Mature odontoblast cells in area of reading.

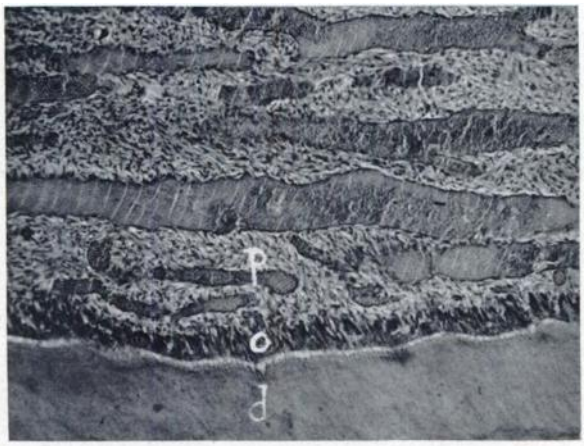


Fig. 5 Incisal end of incisor tooth showing senile odontoblasts. (P) indicates, pulp, (o) odontoblasts, and (d) dentine, respectively.

intake, the odontoblast length bears a logarithmic relation to the vitamin C fed. With intakes of less than 0.5 mg the cells are so disorganized that accurate readings are difficult, and in addition, frank scurvy is present in many individual animals which, in our opinion, renders them unacceptable for assay purposes.

Allotment considerations

We have now conducted tests involving some 650 pigs, aimed at establishing an assay procedure having the obviously desirable characteristics of precision, simplicity and reliability.

In this connection it may be stated that when raised to assay age on our vitamin supplemented basal diet, the absence or use of green feed is apparently immaterial. Further, there are no differences between the sexes in odontoblast cell development and the animals may be penned alone or in groups during the assay. Somewhat more regular curves have been obtained in assays of 35 and 42 days than on shorter periods, and selection of pigs for assay on the basis of age (28 days) has been preferable to the use of an attained live weight of 300 ($\pm$ 5) gm.

Limits of assay range

The response to ascorbic acid of pigs carried under assay conditions has been measured at daily intakes of 0.25, 0.50, 1.0, 2.0, 4.0, and 8 mg. In our experience, pigs carried for over 28 days on intakes of 0.25 mg of ascorbic acid have shown frank scurvy including capillary hemorrhage in the hind legs. No cases of clinical scurvy have been seen on intakes of 0.5 mg per day.

At the upper end of the range we have not found satisfactory increased response with intakes of 4.0 or 8.0 mg over that shown for 2.0 mg. We have not thus far investigated values intermediate between 0.25 and 0.5 mg, or between 2.0 and 4.0 mg of ascorbic acid.

Precision of the assay

In all tests, in addition to the odontoblast readings, the gains of the animals have been recorded and a gain response curve determined. When male animals only are used, the mean estimated potency of the carrier was frequently approximately the same as determined from the odontoblast readings. The response of the females, however, was usually unsatisfactory because of much slower gains.

The relative precision of the 2 methods (gain vs. odontoblast) is striking. The precision of an assay of this type may be expressed as the ratio of the standard error of the mean difference between 2 levels of vitamin intake and the slope of the regression connecting them,

$$pr = \frac{\sigma}{b} \times \sqrt{\frac{2}{n}} \qquad (1)$$

where n is the number in the group, σ the standard deviation of the assay, and pr the precision.

One measure of the relative precision may be indicated by the number of animals needed per group to give the same values for pr. For this purpose we may write the above formula in the form of

$$n = \frac{2}{pr^2} \times \frac{\sigma^2}{b^2} \qquad (2)$$

The number of animals needed per group (i.e., test level group) where growth is the criterion to give a precision (pr) equal to that attained with the number actually used for the odontoblast readings may then be calculated by using in formula (2) the values of pr^2 found for the odontoblast readings and those of σ^2 and b^2 found for the growth values. The n thus found will be compared to the n used in the odontoblast calculations (see column "nc" of table 2).

Another way of indicating relative precision involves a comparison of the precision indexes of the 2 methods. This is shown in the last column of table 2.

The relevant data for 7 separate assays are given in table 2.

In our experience the odontoblast method has consistently given an appreciable increase in precision over the growth method of estimating vitamin C intake.

TABLE 2

Relative precision of growth vs. odontoblasts as criteria of vitamin C intake for guinea pigs.

SUBSTANCE ASSAYED	ODONTOBLAST METHOD				GROWTH METHOD				*nc* [1]	RELATIVE PRECISION
	σ	*b*	*n*	*pr*	σ'	*b'*	*n'*	*pr'*		
Aqueous soln. ascorbic	2.8	32	10	.039	49.3	247	10	.089	53	.44 [2]
Fresh orange juice	3.5	21	10	.074	56.5	61	10	.415	314	.18
Conc. orange juice	5.4	59	6	.053	26.5	135	6	.113	28	.47
Aqueous soln. ascorbic	3.2	21	10	.070	57.6	105	10	.244	122	.29
Fresh orange juice	3.2	18	10	.082	57.6	122	10	.211	66	.39
Synthetic orange juice	3.2	22	10	.065	57.6	94	10	.273	177	.24
Apple juice	3.2	33	10	.044	57.6	192	10	.134	95	.33

[1] *nc* in growth assay to give *pr* equal to odontoblast assay.

[2] *i.e.*, Growth assay has 44% the precision of the odontoblast assay.

Details of assay procedure

The details of the procedure now used in this laboratory are as follows:

Young guinea pigs bred in our own colony and raised from mothers maintained on the basal diet, above described, supplemented with vitamins A, C, D and E plus either hay or greenfeed as available, are at 28 ($\pm$ 3) days of age, allotted at random to individual cages and allowed free access to the pelleted basal diet and water. Vitamins A, D and E in corn oil are administered weekly, by means of a calibrated hypodermic syringe, with needle removed, in amounts to provide the equivalent of 425 I.U. of vitamin A, 48 I.U. of vitamin D and 3 mg of alpha tocopherol daily.

Both the unknown and the control source of vitamin C are fed at each of at least 3 levels of intake, spaced at equal log intervals. We have continued the restriction in allotment that there shall be equal numbers of each sex on each dose level,

and for convenience, have continued the use of individual penning. (Normally not less than 10 animals per dose are used.)

The feeding period for all pigs is 42 days. Since individual penning is employed, different pigs can start and hence complete a trial at different times. This is advantageous not only in the problem of obtaining the needed numbers of animals for assay at any given time, but especially in avoiding the necessity for processing undue numbers on a single day at the end of the feeding period.

Preliminary preparation of the tooth

At the conclusion of its 42-day feeding period each animal is sacrificed by chloroform. Its lower jaw is removed and divided by a vertical incision between the incisors. The exposed portions of the incisor and that portion of the mandible extending beyond the molars are clipped off (thus allowing the fixing agent more easily to reach the pulp of the incisor), and the remainder placed in 10% formalin.

Following a minimum fixation period of 48 hours in 10% formalin, the teeth are washed in 70% alcohol for 24 hours. Decalcification [3] is then carried out in 10% nitric acid, changing the acid every second day. In 36 to 48 hours the unwanted molar and a jaw tissue is readily trimmed away. The tooth is tested with a sharp needle and is removed from the acid when the entire specimen can be pierced easily. Complete decalcification is usually accomplished in 3 to 4 days.

After rinsing in 1 or 2 changes of water, the teeth are placed in 2% potassium alum for 12 hours. This is followed by another rinse in water and a transfer to 5% sodium bicarbonate for 24 hours.

The incisors are then thoroughly washed in running water for 12 to 24 hours, using a washing bobber, and are prepared for embedding in the steps indicated in table 3.

[3] Based on method published by F. W. Gairns. Stain Technology, vol. 19, no. 4, 1944.

The teeth are now orientated for longitudinal sectioning, embedded in new 60°C. paraffin, and cooled.

Cutting. The paraffin block containing the embedded tooth is secured in the microtome jaw and sections removed until the center of the tooth is exposed, i.e., to the point where the pulp cavity ceases to increase in length or width. From the ribbon of sections representing the tooth center, 4 to 6 sections of 8 to 10 microns in thickness are selected for microscopic examination.

TABLE 3

Steps through alcohol to paraffin.

MATERIAL	IN	OUT
10% alcohol [1]	9:00 a.m.	12:00 noon
20% alcohol [1]	12:00 noon	5:00 p.m.
40% alcohol [1]	5:00 p.m.	9:00 a.m.
60% alcohol [1]	9:00 a.m.	12:00 noon
80% alcohol [1]	12:00 noon	5:00 p.m.
Absolute alcohol	5:00 p.m.	9:00 a.m.
Absolute alcohol	9:00 a.m.	10:30 a.m.
Cedarwood oil	10:30 a.m.	12:00 noon
52°C. paraffin	12:00 noon	2:30 p.m.
60°C. paraffin	2:30 p.m.	4:30 p.m.
	changing once	

[1] Phenol may be added to these alcohols up to 6% to impart an elasticity to the tissues, thus facilitating the cutting of thin sections.

Chilling both knife and blocks in ice water for 10 minutes before sectioning increases the ease with which sections are cut.

Staining. Erhlich's acid haematoxylin and the counterstain eosin are employed in ordinary progressive staining.

The odontoblast row of each of the sections on the slide is examined and 5 readings are taken from that section bearing the highest odontoblasts. The average of these is considered to represent the maximum height of the odontoblast cells of that animal.

Measurements are made under 440 magnification by means of an ocular micrometer and the readings are subsequently

converted to microns. Groups of cells of the same height in the central area of maximum odontoblast development are chosen. Measurements are not made in the regions of the dental papilla, nor at the biting end of the tooth where the odontoblasts are seen in their embryonic and senile states, respectively. On levels of ascorbic acid below 1.0 mg the odontoblast development is more irregular and the avoidance of single cell readings is not always possible.

Statistical treatment of the data

The feeding tests are designed to permit standard analysis of the variance of the data and the application of the factorial scheme for isolating individual treatment effects as described by Bliss and Marks ('39). As already mentioned, at least 3 levels of known and unknown are fed. The factorial analysis, however, is applied to the levels of unknown which lie within the limits of the linear response of the known. By this procedure, the difficulty with levels of unknowns which prove to lie outside the limits of linear response is avoided.

ACKNOWLEDGMENTS

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