SOME FACTORS AFFECTING THE BIOASSAY OF VITAMIN C BY THE ODONTOBLAST METHOD

by

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ABSTRACT

Experiments were carried out to determine the effect of factors, other than vitamin C, on the development of odontoblast cells in the guinea pig incisor tooth. Using the "odontoblast" method of vitamin C bioassay, it was shown that guinea pigs may be assigned to experiment at a fixed initial age or a fixed initial weight, the odontoblast development remaining unaffected. Odontoblast development was not different whether the guinea pigs were penned and fed individually or in groups of two.

A natural, an artificial and a fortified source of vitamin C were assayed simultaneously by the "odontoblast" and by the "increase in weight" methods of vitamin C bioassay. The "odontoblast" method was found to afford a bioassay accuracy greater than that afforded by the "increase in weight" method.

Evidence was produced to support the belief that natural food sources of vitamin C are superior to synthetic vitamin C in the stimulation of odontoblast development.

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CONTENTS

assay of the vitamin content of processed foods imperative. Chemical methods of assay, shorter and simpler than biological methods, were employed. Vitamin assays by chemical procedures, however, did not prove to be entirely free of problems, and results from independent laboratories across the country were frequently marked by high variability. As the usefulness of the chemical assay depends on the applicability of its findings to biological problems, it was inevitable that bioassay methods should be called upon to provide necessary checks against the chemical work being done.

In September 1941, the Department of Pensions and National Health invited the Department of Nutrition at Macdonald College to carry out a series of bioassays of vitamin C in dehydrated and concentrated foods. At that time, this laboratory employed a modification of the standard "increase in weight" method for the biological determination of vitamin C, and guinea pigs were the experimental animals used. It was arranged to have the vitamin C in these foodstuffs determined by chemical procedures under the direction of Dr. W.D. McFarlane, Department of Chemistry (Macdonald College).

In this laboratory, Woolsey (1944), while studying

the histological structure of the incisor teeth of guinea pigs on vitamin C bioassay, observed that the height attained by the mature odontoblast cells is directly dependent on the amount of vitamin C ingested by the animal. On the basis of this relationship, an objective bioassay procedure was developed. The influence of factors other than vitamin C, which might be suspected of affecting odontoblast development were then investigated.

Hughes (1945) continuing the study of this procedure, determined as a result of her experiments in this laboratory, the combined effect of the diet of the mother during pregnancy and lactation and the diet of the progeny during the pre-experimental period, on the development of the odontoblast cells in the young animals during the experimental period. Guinea pigs were also fed individually and in groups so that it might be shown whether or not feeding management influences odontoblast development. In addition, Miss Hughes determined the minimum length of time necessary to complete a satisfactory odontoblast bioassay.

This thesis will report further investigations into the effect of factors, other than vitamin C, on the development of odontoblast cells in the guinea pig incisor tooth.

These investigations were conducted to compare the odontoblast development attained by guinea pigs,

(a) group-fed and individually fed

(b) assigned to assay at a fixed initial age and at a fixed initial weight.

The experiments were designed to permit also a comparison of

(a) the vitamin C bioassay accuracy afforded by the "odontoblast" method with that afforded by the "increase in weight" method, and

(b) the results obtained from each of these biological assays of vitamin C with those obtained from the chemical assay of vitamin C.

LITERATURE REVIEW

The principle on which the biological determination of vitamin C is founded is the belief that the cure of, or, the degree of protection from scurvy, bears a quantitative relationship to the amount of vitamin C ingested by the experimental animal.

Man, monkey and the guinea pig are the only animals that are unable to synthesize their requirements of vitamin C. Hence, guinea pigs, available in large numbers and relatively inexpensive to maintain, are the most widely used animal for the biological determination of the antiscorbutic vitamin.

As early as 1907, Holst and Frolich made comparisons of the antiscorbutic properties of foods by finding whether or not the arbitrarily-chosen quantities which they fed would suffice to prevent the appearance of scurvy in young guinea pigs. The basal diet they employed was deficient in many respects as well as in antiscorbutic vitamin.

Realizing that a successful measurement of the antiscorbutic vitamin depends on the adequacy of the

basal ration, Cohen and Mendel (1918), Chick, Hume and Skelton (1918), Delf (1918) and others, devised new diets planned to provide all nutritional factors (except the antiscorbutic) then known to be necessary for normal growth of the guinea pig.

The work of Holst and Frolich (1907), however, did not attract much attention until the substance in food, the absence of which causes scurvy, was placed among the vitamin group as vitamin C by Drummond (1919). A new interest in scurvy then became evident in the scientific literature of America and England.

Sherman, working with La Mer and Campbell (1922), measured relative amounts of vitamin C by determining how much of the antiscorbutic food substance was required to prevent scurvy in guinea pigs of 300 grams initial weight, for a period of 90 days. This amount was called the minimum protective dose. When less than this dose was fed, the antiscorbutic vitamin was measured by a quantitative rating of the severity of the scurvy produced. This rating was based on the weight curve, the survival period and the severity of ante- and post-mortem symptoms of scurvy in the assay guinea pigs.

These workers developed a diet for guinea pigs which was markedly superior to the hay-oats-water combination previously used to produce experimental scurvy. Hojer (1924) working with Sherman's method, remarked, "Even with these improvements ....., this method of determining the fully protective dose is very inaccurate and often gives variations of 100% or more."

Harris and Ray (1932) performed biological assays of vitamin C using the curative method. Male guinea pigs weighing about 250 grams were given, for a preliminary period of 10 days, a scorbutic diet supplemented by 15 grams of cabbage per day. In the following period of 2 to 3 weeks, the animals were fed the basal diet alone. Guinea pigs which were chosen for test showed early symptoms of scurvy and had begun to lose weight evenly, dropping sharply 10 to 20 grams from their maximal weights in the course of three days. Graded daily doses of the antiscorbutic supplement were fed and the rate of recovery of body weight noted. The doses of unknown and of standard needed to cause equal resumption in growth rate were compared and a smooth response curve, relating increase in weight to dose of material, was obtained.

Curative methods of vitamin assay, however, have fallen from general use. In estimating the end of the depletion period, there is room for a variability which may exert a powerful influence on the results obtained. Many workers have observed that there is frequent failure of the animal, after it has ceased to grow, to respond to even relatively large doses of a vitamin with anything approaching normal growth.

Coward and Kassner (1936) worked out a preventative method for the estimation of vitamin C on the basis of its influence on the body weight of guinea pigs. Today, this method is accepted by most workers as a standard procedure for the determination of vitamin C when increase in weight is used as the criterion of vitamin intake. Coward and Kassner fed different groups of guinea pigs daily doses of 0.125, 0.25, 0.5, 1.0 and 2.0 mg. of ascorbic acid (successfully synthesized by Haworth (1933)). Each pig in any group received the same daily dose. A diet containing an abundance of all other substances known to be necessary for growth was given ad libitum. The guinea pigs were weighed twice a week. When the test was carried on for six weeks a fairly smooth curve of response was obtained, relating increase in weight to dose

of vitamin C given.

Side by side with the development of the "increase in weight" method for the biological determination of vitamin C, another method for the determination of this vitamin was evolving, based on the histological change occurring in the teeth of young guinea pigs fed scorbutogenic diets.

It was through the histological studies of Zilva and Wells (1919) that the tooth was identified as the first of the guinea pig tissues to be affected by vitamin C deficiency.

Some years later, Hojer (1924, 1926) observed that the degrees of histological change in the tooth of the guinea pig depends on the amount of vitamin C made available to the animal. Examining transverse sections, he made detailed descriptions of ten stages in the development of scorbutic lesions in the teeth of guinea pigs which had received doses of vitamin C graded from nil to completely protective. Hojer referred to these descriptions as a scale upon which to determine the antiscorbutic value of foodstuffs. He expressed the degree of tooth protection afforded by any dose as a fraction of complete protection and assumed this fraction to be the ratio of

the test dose to the minimum dose giving complete tooth protection.

Goettsch (1928), following Hojer's procedure, found it difficult to subjectively define the fractions of the minimum protective dose due to the variable response of individual animals in any one group. In comparing the potency of two materials, she determined the minimum dose of each that would give complete protection, making no use of the responses of animals receiving less than the minimum protective dose.

Three years later, Key and Elphick (1931) published a method for testing the antiscorbutic potency of foodstuffs based on the work of Hojer and Goettsch. Using sufficient numbers of animals to eliminate differences due to individual variation, they set a numerical scale where the figures from 0 to 4 represented five different histological pictures appearing with the development of scorbutic symptoms in the tooth. Each tooth examined was assigned to one of these five groups and given its corresponding numerical value. These workers were able to obtain a straight line response when the degree of protection from scurvy was plotted against the dose of the standard.

Coward and Kassner (1936), comparing the precision of their "increase in weight" method with that of the "tooth"

method as revised by Key and Elphick, noted,

> "..... 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."

> "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".

" ..... 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 ".

Harris and Ray (1933) and Fish and Harris (1935) were the first to observe that in the estimation of vitamin C by the "tooth" method, the histological picture that appears in cross-section is greatly affected by the level at which the tooth is cut. Heretofore, all "tooth" methods had made use of transverse sections, but Harris now points out that certain characteristics of the guinea pig tooth render the use of longitudinal sections necessary. The teeth of the guinea pig, both incisors and molars, are of persistent growth. In any tooth at a given moment, the ameloblasts, cementoblasts, odontoblasts and other cells may, therefore, be observed in an embryonic state, in a state of maturity, or, again, in a condition of senility and degeneration.

In all "tooth" methods the state of odontoblast development and organization has been regarded as a reliable indication of the degree of scorbutus; yet, as

the odontoblasts move up with the persistently growing tooth, they pass through every phase of their life history. At the growing end of the tooth they may be seen differentiating from the embryonic mesoderm cells of the papilla. Midway of the pulp length they are typical odontoblasts --- cylindrical cells, each possessing a pronounced fibril running into a tubule of the dentine. At the older end of the pulp they are degenerating. In full scurvy an acceleration of this process of degeneration appears to take place. Not only do the odontoblasts at the senile end of the tooth die, but the younger odontoblasts all the way down the pulp also degenerate and lose their fibrils. In subclinical scurvy, some of the youngest of the odontoblasts remain alive for a time. They soon begin to degenerate, however, and lose their fibrils. At the senile, biting end of a normal tooth, therefore, a condition may be observed very much the same as is seen lower down in the pulp in scurvy.

This work of Fish and Harris (1935) has shown that a reliable assessment of the dental tissues is impossible except when longitudinal sections are studied. No record has been found in the literature of the use of longitudinal sections by other workers assaying vitamin C by the "tooth"

method, until Woolsey (1944), working in this laboratory, studied from longitudinal sections the effect of varying intakes of vitamin C on the guinea pig tooth.

Woolsey determined the effect of graded intakes of ascorbic acid on the height of the odontoblast cells in the guinea pig incisor tooth. (Zilva and Wells (1919) had regarded these cells as the first to exhibit change in a tooth affected by scurvy). Woolsey measured micrometrically the maximum height of the mature odontoblasts appearing in a section taken lengthwise through the centre of the guinea pig incisor tooth.

He found the degree of odontoblast development to be dependent on the amount of ascorbic acid ingested by the animal. Six equally graded doses of crystalline ascorbic acid, ranging from 0.25 to 8.0 mg. were fed daily. The odontoblast responses to intakes of 0.5, 1.0 and 2.0 mg. daily, were shown by statistical analysis to be significantly different, and within this range of intakes, the relationship of mature cell heights to the logarithm of the daily dose was linear. The odontoblast cells showed no increased response to intakes of ascorbic acid greater than 2.0 mg. daily, and on intakes of less than 0.5 mg. daily, the abnormal, unmeasurable odontoblasts associated with subclinical scurvy were observed.

The establishment of the relationship between odon-

toblast cell height and ascorbic acid intake made possible the quantitative determination of vitamin C by an objective bioassay procedure.

Working in this laboratory, Hughes (1945) used the odontoblast method with success in determining the vitamin C potency of a concentrated foodstuff, a dehydrated foodstuff and a solution of crystalline ascorbic acid of 'unknown' potency supplied by the Department of Pensions and National Health, Ottawa. The "increase in weight" method of Coward and Kassner (1936) was used simultaneously with the "odontoblast" method. Although the coefficient of variability associated with the "increase in weight" method was found to be 33%, that associated with the "odontoblast" method was found to be 13%.

Miss Hughes also conducted experiments to study the effect of factors, other than vitamin C, on the development of the odontoblast cell. At the conclusion of a 42-day assay period, she was able to show that the diet of the mother during pregnancy and lactation and the diet of the progeny during the pre-test period, had no measurable effect upon the development of the odontoblast cells of the progeny during the experimental period.

Through her work it was learned that guinea pigs may be penned and fed individually or in groups of two,

the odontoblast development remaining unaffected.

Miss Hughes, in another experiment, determined the minimum length of time necessary for the completion of a satisfactory "odontoblast" assay to be 35 days. The persistently-growing guinea pig tooth, however, requires 40 days to complete one full cycle of growth (Fish and Harris, 1935). Hence, using the "odontoblast" method, one is assured at the end of a 42-day assay period that the height of the odontoblast cells has been influenced only by the experimental treatments imposed.

A diet suitable for use in vitamin C bioassay work should, theoretically, supply all the nutritional requirements of the guinea pig excepting ascorbic acid. Working at Macdonald College, Farmer (1944) was unable to devise a diet which was completely satisfactory for normal reproduction of the guinea pig unless green-stuffs were also supplied. These results were recently confirmed by Bell (1946). Greenfeeds, however, contain variable quantities of vitamin C and, for this reason, cannot be included in diets which are to be used for the bioassay of vitamin C.

The ration (Macdonald College Diet No.5) developed in this laboratory permits about 80% successful reproduction

when fortified with fresh greens, yet has a reproduction efficiency of 70% when unsupplemented by greenstuffs. Macdonald College Diet No.5 when supplemented with crystalline vitamin C, however, promotes in guinea pigs a steady uniform rate of growth to maturity. This ration has been found to be an improvement over any of the guinea pig diets reported in the literature (Farmer, 1944) and is fed to guinea pigs assigned to vitamin C bioassay in this laboratory.

EXPERIMENTAL PROCEDURE

In an effort to obtain further information concerning the bioassay of vitamin C by the "odontoblast" method, two experiments, involving a total of 220 guinea pigs, were carried out. A 42-day assay period was allowed for each experiment.

a. Methods of Assay.

The "increase in weight" method for the biological determination of vitamin C, developed by Coward and Kassner (1936), was applied to each experiment simultaneously with the "odontoblast" method. The vitamin C potencies of the food substances assayed by the two biological methods, were also determined by chemical procedures.

b. Animals.

The guinea pigs were progeny of the colony maintained at Macdonald College. When randomized to an experiment, the animals were assigned to roomy, all metal cages with perforated floors. Ad libitum feeding was permitted and fresh water was supplied daily.

c. Diet

The formula, compiled in this laboratory, for the basal diet fed throughout both experiments, is given below.

Macdonald College Diet No. 5

Ground oats	.....	15.0%
Ground wheat	.....	13.0%
Beet pulp	.....	25.0%
Linseed oilmeal	.....	12.5%
Skimmilk powder	.....	15.0%
Fishmeal	.....	5.0%
Dried brewers' yeast	..	10.0%
Bone char	.....	4.0%
Salt	.....	0.5%

These ingredients are ground, mixed and pressed into pellets 3/16 of an inch in length and 1/8 of an inch in diameter. Guinea pigs readily accept the pellet diet, whereas they often find feeds of a powdery nature unpalatable.

d. Supplements

All supplements were administered orally to the guinea pigs by means of a graduated tuberculin syringe.

Vitamin C

The standard supplement against which all other antiscorbutic substances were measured, was pure, crystalline vitamin C. Just before feeding, the pure vitamin was dissolved in distilled water.

Vitamins A, D and E

Vitamins A, D and E were administered weekly as a mixture of ling liver oil, calciferol and alphatocopherol, in corn oil. A weekly dose of 0.4 c.c. of this solution was, for each animal, equivalent to a daily intake of 425 I.U. of vitamin A, 48 I.U. of vitamin D_2 and 3 mg. of vitamin E.

e. Records

Throughout the 42-day term of experiment, a record was kept of the feed consumed and the weight gained by each animal during each seven-day period. At the conclusion of the experiment, the general condition of each guinea pig was checked macroscopically by post-mortem examination.

f. Statistical Analysis of Experimental Data

All experimental data were examined statistically by the method of Bliss and Marks (1939). The design of each experiment and the partition of variance within each experiment appear in Appendix I.

g. Histological and Micrometric Technique

The technique employed in the preparation of the incisor teeth for histological examination and the method followed in making microscopical measurements of odontoblast cell height are described in Appendix II.

EXPERIMENTAL RESULTS

The Effect of Individual and Group Methods of Feeding on Odontoblast Development

Hughes (1945), working with the "odontoblast" method of vitamin C bioassay, reported that the height attained by the odontoblast cell is not different whether the guinea pigs are penned and fed individually or in groups of two.

As part of the later work done on the "odontoblast" method, this thesis will report an experiment involving 160 animals. According to the design of this experiment, illustrated in Table 1, four of each group of ten animals were penned in groups of two and six were penned individually. For a large number of animals, such a penning arrangement is economical of space and labor. The plan, however, was useful primarily in that it provided an opportunity to check the results obtained by Hughes.

The effect of individual feeding and of group feeding on the height of the odontoblast cells in the guinea pig incisor tooth is illustrated in Table 2. It will be noted that at the end of a six weeks' test, no difference in the odontoblast development of individually-fed and group-fed

animals could be demonstrated. These findings are in agreement with those reported by Hughes (1945).

TABLE 1. ANIMAL ALLOTMENT PLAN

Level of Ascorbic Acid (mg.)	Feeding Management	Sex	Control Ascorbic Acid	Assay Material (A)	Assay Material (B)	Assay Material (C)
0.5	Group Penning	♂	2 pigs			
		♀	2 pigs			
	Individual Penning	♂	3 pigs			
		♀	3 pigs			
1.0	Group Penning	♂		2 pigs		
		♀		2 pigs		
	Individual Penning	♂		3 pigs		
		♀		3 pigs		
2.0	Group Penning	♂			2 pigs	
		♀			2 pigs	
	Individual Penning	♂			3 pigs	
		♀			3 pigs	
4.0	Group Penning	♂				2 pigs
		♀				2 pigs
	Individual Penning	♂				3 pigs
		♀				3 pigs

TABLE 2. EFFECT OF INDIVIDUAL AND GROUP METHODS OF FEEDING ON ODONTOBLAST CELL HEIGHT

Level of Ascorbic Acid (mg.)	Feeding Management		Observed Difference
	Individual Feeding	Group Feeding	
	odontoblast cell heights (microns)		
0.5	21.1	21.6	0.5
1.0	29.1	28.5	0.6
2.0	35.4	35.3	0.1
4.0	35.8	35.5	0.3

$$\text{Necessary Difference} = \sqrt{\frac{10.33}{24} + \frac{10.33}{16}} \times t_{.05} = 2.08 \text{ microns}$$

The Effect on Odontoblast Development of Assigning Guinea Pigs to Assay at a Fixed Initial Weight or at a Fixed Initial Age.

An experiment was carried out to determine whether or not, in the bioassay of vitamin C by the "odontoblast" method, there is a significant relationship between either initial age or initial weight and odontoblast height.

Two trials were conducted, the designs for which are shown in Tables 3 and 4. In the first trial guinea pigs were assigned to experiment at ages ranging from 34 to 41 days. In the second trial all animals were assigned to experiment at weights ranging from 295 to 305 grams. The weights of the guinea pigs assigned to assay at a fixed initial age ranged from 285 to 440 grams. The ages of the animals assigned to assay at a fixed initial weight ranged from 15 to 35 days.

At the conclusion of both six-week trials, the correlation existing between initial age and odontoblast height and between initial weight and odontoblast height was statistically determined.

TABLE 3. DESIGN OF TRIAL I IN WHICH GUINEA PIGS WERE ASSIGNED TO ASSAY AT FIXED INITIAL AGE

Level of Ascorbic Acid (mg.)	Sex	Initial Weights of Guinea Pigs Assigned to Assay at Fixed Initial Age (grams)	
		Control Ascorbic Acid	Assay Material
0.5	♂	346 289 400 349 370	383 344 343 349 407
	♀	310 295 324 313 354	326 347 344 340 249
1.0	♂	378 365 364 308 380	340 345 335 308 385
	♀	308 309 358 313 315	350 293 330 309 373
2.0	♂	385 351 351 286 306	378 335 440 430 359
	♀	329 333 325 369 358	360 302 306 286 370

TABLE 4. DESIGN OF TRIAL II IN WHICH GUINEA PIGS WERE ASSIGNED TO ASSAY AT FIXED INITIAL WEIGHT.

Level of Ascorbic Acid (mg.)	Feeding Management	Sex	Initial Ages of Guinea Pigs Assigned To Assay At Fixed Initial Weight. (days)			
			Control Ascorbic Acid	Assay Material (a)	Assay Material (b)	Assay Material (c)
0.5	Group Penning	♂	29	31	18	19
		♂	29	22	24	19
		♀	27	30	30	33
		♀	28	30	21	32
	Individual Penning	♂	20	24	19	25
		♂	26	22	35	20
		♂	31	30	21	21
		♀	27	27	27	28
		♀	27	24	30	20
		♀	30	20	25	19
1.0	Group Penning	♂	15	17	21	19
		♂	30	16	24	20
		♀	21	25	32	17
		♀	21	27	37	26
	Individual Penning	♂	21	21	18	24
		♂	33	23	21	20
		♂	21	26	31	29
		♀	23	31	27	26
		♀	29	29	28	20
		♀	28	23	17	19
2.0	Group Penning	♂	22	26	23	15
		♂	27	28	25	25
		♀	21	31	20	18
		♀	35	35	21	24
	Individual Penning	♂	28	25	21	24
		♂	26	20	26	21
		♂	23	22	15	31
		♀	20	23	30	27
		♀	26	29	17	29
		♀	23	20	21	37
4.0	Group Penning	♂	31	28	16	22
		♂	20	23	16	20
		♀	24	27	29	24
		♀	30	32	30	26
	Individual Penning	♂	21	24	18	18
		♂	23	15	25	24
		♂	20	26	26	26
		♀	18	21	19	20
		♀	22	20	21	24
		♀	20	20	21	30

The analyses of covariance between initial age and odontoblast height and between initial weight and odontoblast height are presented in Tables 5 and 6 respectively. It will be seen that these trials have shown no significant correlation to exist between either initial age (when initial weight lay within the range of 285 to 440 grams) and odontoblast height, or initial weight (when initial age lay within the range of 15 to 35 days) and odontoblast height. These results would indicate that, in the assay of vitamin C by the "odontoblast" method, guinea pigs may be assigned to assay at either a fixed initial age or a fixed initial weight.

Table 5. Analysis of Covariance Between Initial Age and Odontoblast Height

Source of Variance	D/F	$S(x-\bar{x})(y-\bar{y})$	Covariance	r
All causes	159	-239.7		
Between subgroups	63	-512.1		
Remainder	96	272.4	2.84	+0.1566

For 96 degrees of freedom, at the 5% point, an r value of 0.1946 is required for significance.

Table 6. Analysis of Covariance Between Initial Weight and Odontoblast Height

Source of Variance	D/F	$S(x-\bar{x})(y-\bar{y})$	Covariance	r
All causes	59	708.5		
Between subgroups	11	1533.1		
Remainder	48	-824.6	-17.2	0.1468

For 48 degrees of freedom, at the 5% point, an r value of 0.2372 is required for significance.

THE DETERMINATION OF THE VITAMIN C POTENCY OF A NATURAL, A SYNTHETIC AND A FORTIFIED SOURCE OF VITAMIN C BY THE "ODONTOBLAST" METHOD AND BY THE "INCREASE IN WEIGHT" METHOD.

The work of Elmby and Warburg (1937) and unpublished assay work done in this laboratory have indicated that vitamin C in natural foods is of greater biological value than an equivalent amount of vitamin C supplied as crystalline ascorbic acid. A factor (or factors) has been postulated to be present in natural sources of vitamin C, which acting either synergistically with or independently of vitamin C, enhances the biological value of the foodstuff. Elmby and Warburg have suggested that this factor may be vitamin P.

An experiment was carried out in this laboratory to further investigate the apparent enhanced biological value of natural sources of vitamin C. The design of this trial appears in Table 7. The experiment, involving 160 guinea pigs, provided for the determination of the antiscorbutic values of food substances by simultaneous measurement of odontoblast response in the guinea pig incisor tooth and growth response as manifested by increases in live weight.

TABLE 7. EXPERIMENTAL DESIGN FOR THE DETERMINATION OF THE VITAMIN C POTENCY OF A NATURAL, A SYNTHETIC AND A FORTIFIED SOURCE OF VITAMIN C.

Level of Ascorbic Acid (mg.)	Feeding Management	Sex	Mean Height Attained by the Odontoblast Cells of Each Animal (microns)			
			Control Ascorbic Acid	Fresh Orange Juice	Artificial Orange Juice	Canned Fortified Apple Juice
0.5	Group Penning	♂	25	25	23	19
		♂	22	16	19	15
		♀	26	26	25	17
		♀	22	28	21	17
	Individual Penning	♂	24	28	28	20
		♂	18	25	20	14
		♂	25	24	23	17
		♀	20	22	25	15
		♀	23	23	20	20
		♀	15	22	20	16
1.0	Group Penning	♂	21	29	27	29
		♂	19	30	26	27
		♀	32	32	29	29
		♂	24	32	29	32
	Individual Penning	♂	29	36		
		♀	30	30	30	28
		♀	37	35	32	21
		♀	30	30	29	17
2.0	Group Penning	♂	32	40	30	39
		♂	34	35	34	34
		♀	33	32	41	31
		♀	37	30	39	45
	Individual Penning	♂	44	34	35	36
		♂	33	35	35	32
		♂	33	33	38	36
		♀	32	33	38	37
		♀	30	38	32	39
		♀	36	37	38	39
4.0	Group Penning	♂	40	38	38	34
		♂	35	36	38	35
		♀	32	37	35	37
		♀	32	34	35	35
	Individual Penning	♂	36	42	35	36
		♂	36	34	34	34
		♂	38	39	33	38
		♀	35	32	32	39
		♀	36	34	42	32
		♀	36	32	38	39

To make this test, three assay materials were selected. Freshly expressed orange juice was selected as a common natural source of vitamin C. An artificial orange juice, made up according to the formula developed by Radford, de Savitsch and Sweany (1937), was chosen as a synthetic material comparable in composition and pH to fresh orange juice. This material has no antiscorbutic value. Its vitamin C potency was determined by the addition of crystalline ascorbic acid.

Formula of Radford, de Savitsch and Sweany

Buffer mixture	74	grams
Sugar	396	"
Orange extract	5	c.c.
Water to make	125	oz.

The buffer mixture consists of:-

Calcium phosphate: $CaHPO_4.H_2O$	1.075%
Calcium citrate: $Ca_3(C_6H_5O_7)_2.4H_2O$	4.57
Sodium phosphate: Na_2HPO_4	3.83
Citric acid: $C_6H_8O_7.H_2O$	59.08
Sodium citrate: $Na_3C_6H_5O_7.2H_2O$	12.75
Corn starch	18.62
Sunset yellow	0.05

The orange extract is a solution of 4.75% orange oil plus 95.25% of 95% ethyl alcohol.

Canned fortified apple juice* was selected as a substance which contains vitamin C partly in natural combination and partly in the form of the synthetic vitamin. These three materials were assayed against an aqueous solution of standard crystalline ascorbic acid.

The natural juices were analyzed chemically for total ascorbic acid according to the method of Roe and Kuether (1943) and for dehydroascorbic acid according to the method of Roe and Oesterling (1944).

The curves of odontoblast response to the materials assayed are shown in Figures I and Ia. The curves of gain response are shown in Figures II and IIa.

* A product prepared by S. Allen Ltd., Norwich, Ontario.

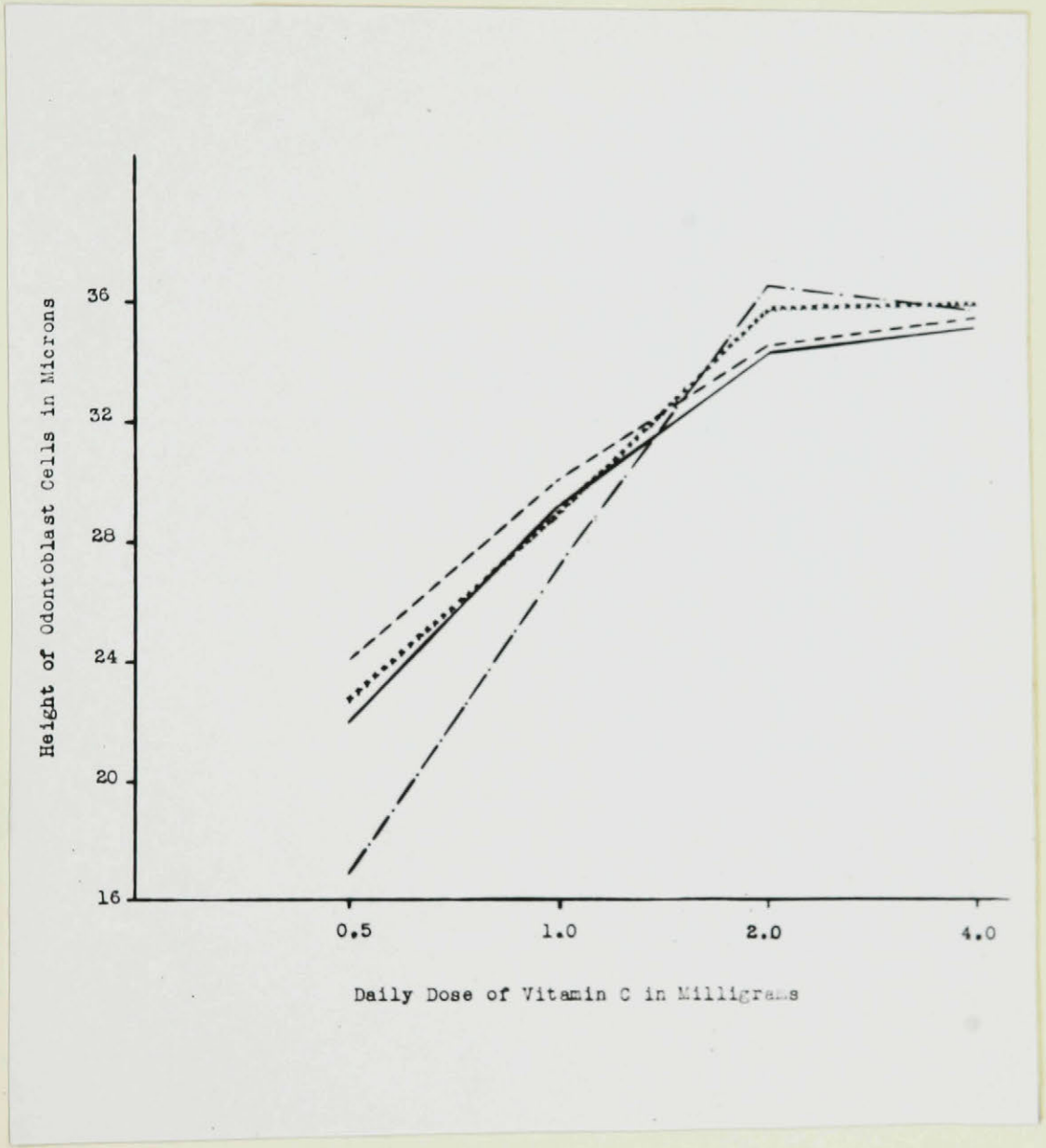

Figure I. Observed Odontoblast Response to Graded Doses of Vitamin C, Supplied as:

_____ Ascorbic Acid

_ _ _ _ _ Fresh Orange Juice

xxxxxxxxx Artificial Orange Juice

-.-.-.-.-. Fortified Apple Juice

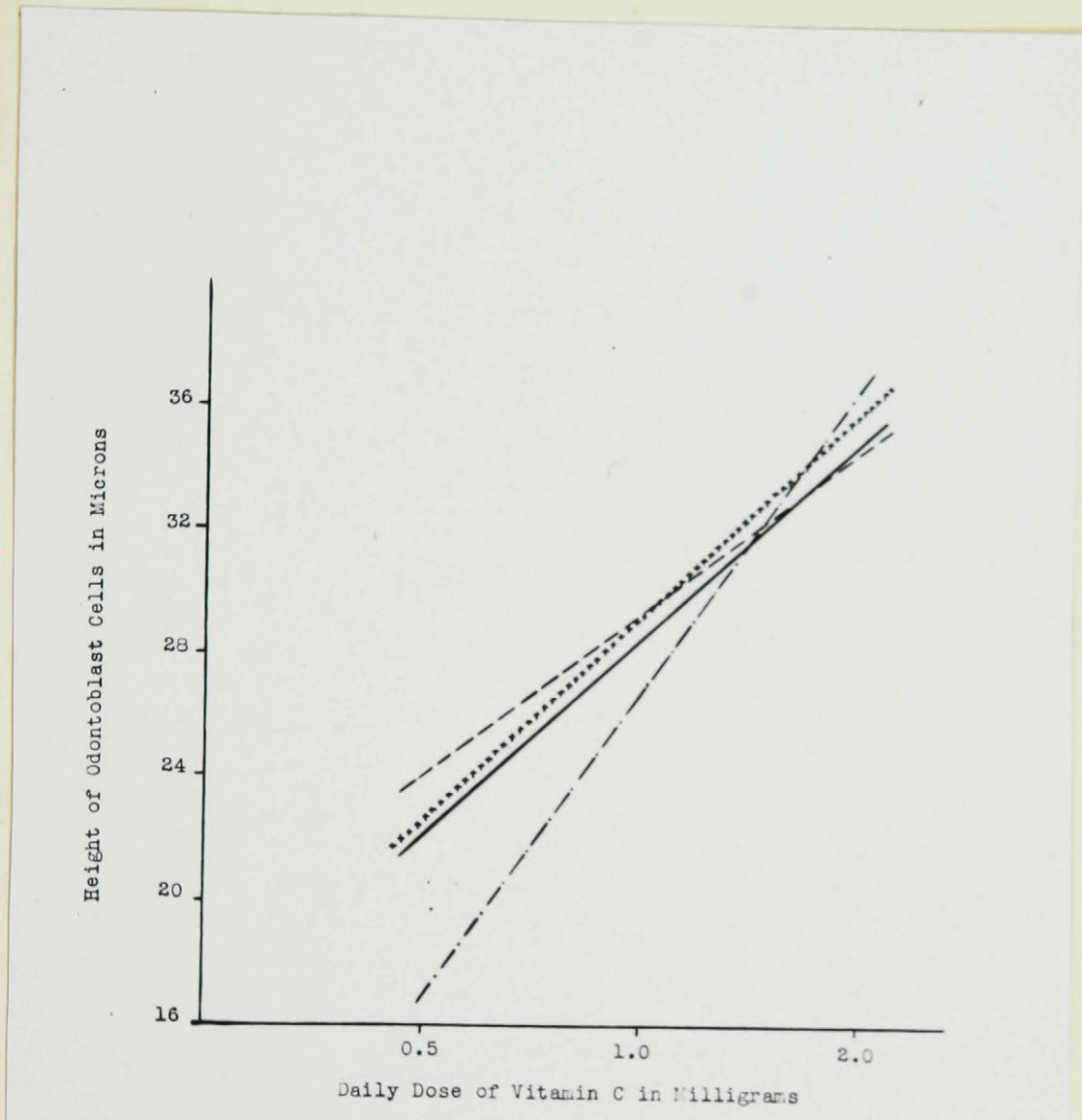

Figure Ia. Regression of Height of Odontoblast Cells on Daily Vitamin C Intake, Supplied as:

_____	Ascorbic Acid	Y = 7.7 + 20.8 (log. 10 dose)
_ _ _ _	Fresh Orange Juice	Y =11.8 + 17.6 (log. 10 dose)
xxxxxxx	Artificial Orange Juice	Y = 6.9 + 22.2 (log. 10 dose)
.-.-.-.	Fortified Apple Juice	Y =-6.0 + 32.9 (log. 10 dose)

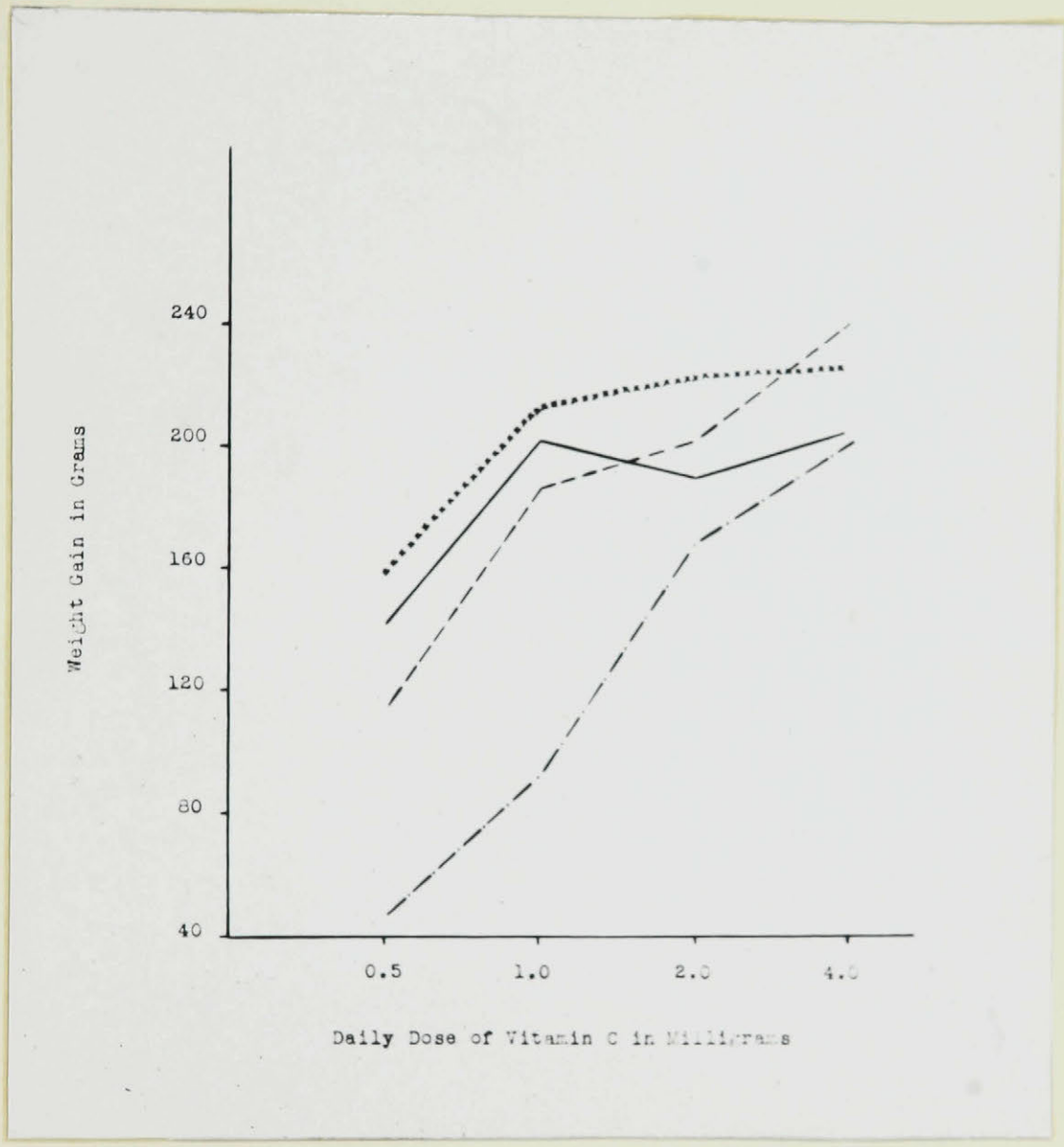

Figure II. Observed Gain Response to Graded Doses of Vitamin C, Supplied as:

_____ Ascorbic Acid

_ _ _ _ Fresh Orange Juice

xxxxxxx Artificial Orange Juice

_ . _ . Fortified Apple Juice

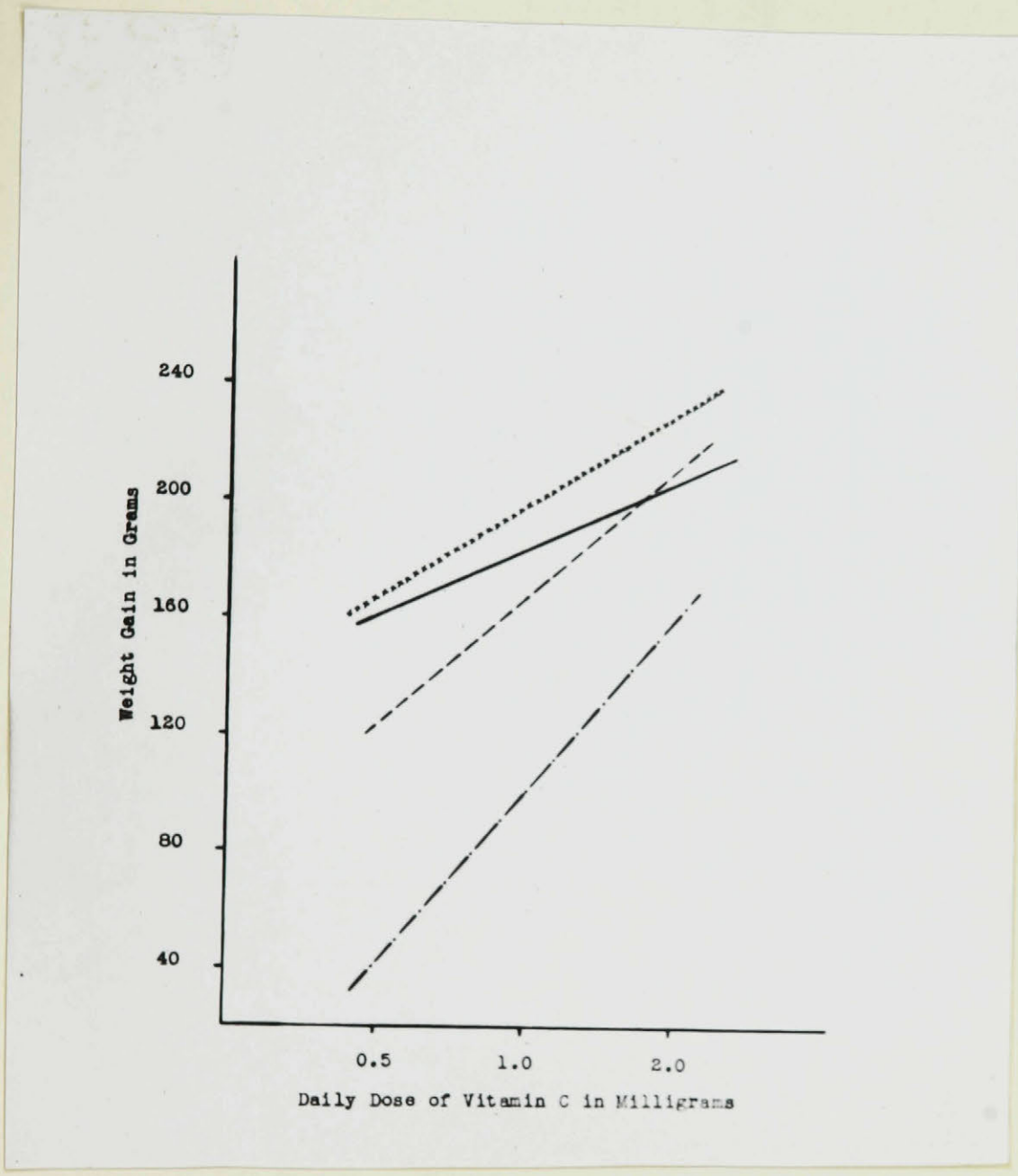

Figure IIa. Regression of Live Weight Increases to Graded Doses of Vitamin C, Supplied as:

_____	Ascorbic Acid	Y = 109.4 + 76.1 (log. 10 dose)
_ _ _	Fresh Orange Juice	Y = 21.6 + 146.9(log. 10 dose)
xxxxxx	Artificial Orange Juice	Y = 90.1 + 108.0(log. 10 dose)
-.-.-.	Fortified Apple Juice	Y = -98.3 + 200.0(log. 10 dose)

Table 8 gives the apparent antiscorbutic value of each assay material at each level at which it was fed, as measured from the odontoblast and the growth response curves.

Table 8. Apparent Antiscorbutic Value of Assay Materials at Each Level Fed as Measured from the Odontoblast and Gain Response Curves.

Assay Material	Daily Dose of Ascorbic Acid Fed (mg.)	Apparent Ascorbic Acid Intake (mg.)	
		Odontoblast Response Curve	Gain Response Curve
Fresh Orange Juice	0.5 1.0 2.0 4.0	0.6 1.2 2.0 2.2	0.1 1.0 1.7 5.2
Artificial Orange Juice	0.5 1.0 2.0 4.0	0.5 1.0 2.2 2.3	0.4 2.4 3.1 3.3
Fortified Apple Juice	0.5 1.0 2.0 4.0	0.3 0.9 2.4 2.2	-0.69 -0.17 0.6 1.6

In Table 9 are presented the vitamin C potencies of the materials assayed as determined by both biological and chemical methods.

Table 9. SUMMARY OF BIOLOGICAL AND CHEMICAL ESTIMATES OF POTENCY

Assay Material	Average Ascorbic Acid Content (mg. per 100 ml.)			
	Chemical Assay	Odontoblast Response Curve	Odontoblast Assay (Bliss and Marks' Analysis)	Growth Response Curve
Fresh Orange Juice	56.0	56.0	45 - 70	43
Artificial Orange Juice	50.0	53.0	44 - 64	70
Fortified Apple Juice	37.0	52.0	43 - 64	17

The precision of an experiment may be measured by the ratio of the standard error of the mean difference to the regression or slope. Hence, a steeper slope and/or a smaller standard error will give a smaller ratio which is indicative of increased precision. A comparison of the experimental precision afforded by the "odontoblast" and "growth" methods of vitamin C bioassay is presented in Table 10.

It will be observed in this experiment that the precision of the "odontoblast" method is three times that of the "growth" method. This is not surprising when it is realized that the measurement of odontoblast response is a measurement of a specific effect on a specialized tissue, whereas the measurement of growth response is a measurement of a general effect on all tissues.

Table 10. PRECISION OF THE "ODONTOBLAST" AND "GROWTH" METHODS OF VITAMIN C BIOASSAY

Assay Material	Ratio of the Standard Error of the Mean Difference to the Slope	
	Odontoblast Assay	Growth Assay
Control Ascorbic Acid	0.070	0.244
Fresh Orange Juice	0.082	0.211
Synthetic Orange Juice	0.065	0.273
Fortified Apple Juice	0.044	0.134

DISCUSSION

The odontoblast response to supplements of natural orange juice, artificial orange juice, fortified apple juice and crystalline ascorbic acid is graphically illustrated in Figure I, page 36.

When the odontoblast response to the 0.5 mg. and 1.0 mg. levels of intake is studied, fresh orange juice appears to promote a greater odontoblast development than that promoted by an equivalent amount of crystalline ascorbic acid in watery solution. At the 2.0 mg. level of intake, however, the value of natural orange juice and crystalline ascorbic acid appear to be identical.

It must be considered that, in this, as well as in all assays conducted by Woolsey (1944) and Hughes (1945), the odontoblasts have shown no increased response to crystalline ascorbic acid when it is fed in amounts exceeding 2.0 mg. daily. If odontoblasts, stimulated by natural sources of vitamin C, attain a higher development than odontoblasts stimulated by equivalent amounts of synthetic vitamin C, then an intake of less than 2.0 mg. of the natural vitamin carrier will induce the maximum odontoblast development for which 2.0 mg. of the crystalline vitamin is required.

Figure III illustrates how the superior value of a natural vitamin-C-containing substance might influence the odontoblast response curve.

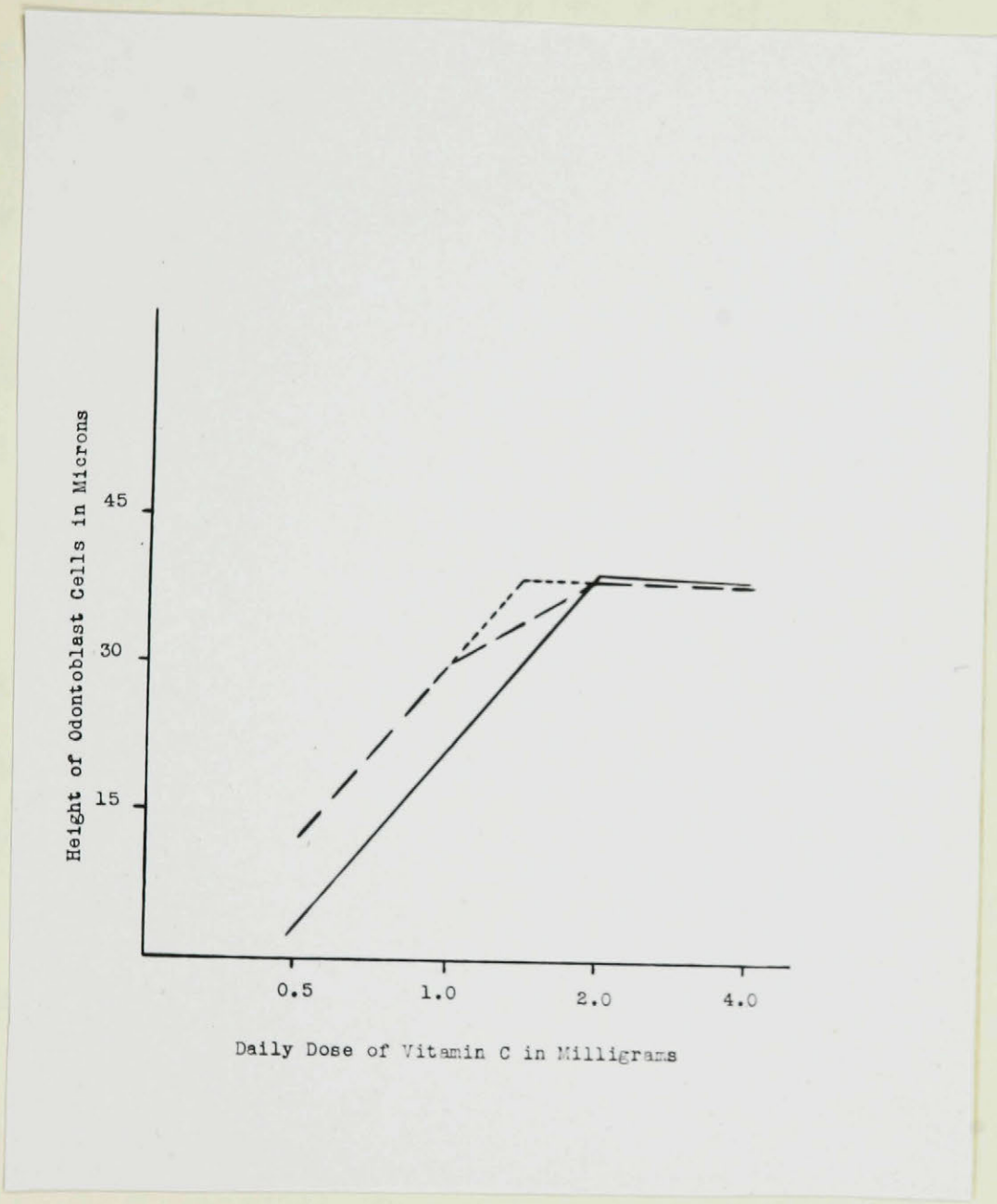

Figure III Odontoblast Response Curves to Graded Doses of Ascorbic Acid, Supplied as:

———— Ascorbic Acid

— — — Natural Source of Vitamin C

- - - - - - Hypothetical Odontoblast Response to Natural Source of Vitamin C Fed at a Level Intermediate Between 1.0 mg. and 2.0 mg.

It is probable that the curve of odontoblast response to a natural antiscorbutic reaches its highest point somewhere between the 1.0 mg. and 2.0 mg. levels of intake. It is impossible, therefore, to determine this point of maximum odontoblast response unless the natural vitamin C medium is fed in a dose intermediate between 1.0 mg. and 2.0 mg. When reading the odontoblast response to a 2.0 mg. dose of a natural source of vitamin C, it is probable that we are reading a response curve which has already fallen from its maximum height. An estimate of the biological value of the natural vitamin C source, made at this point, will obviously be in error.

As fresh orange juice proved to be superior to crystalline ascorbic acid on the 0.5 mg. and 1.0 mg. levels of intake, it is suggested that, if assayed at a level between 1.0 mg. and 2.0 mg., fresh orange juice, at this higher level, would also show a superiority over synthetic vitamin C in the stimulation of odontoblast response.

The lowered response to the 0.5 mg. and 1.0 mg. levels of fortified apple juice is attributable to the fact that the amounts of this supplement fed were insufficient to protect the guinea pigs from scurvy. The commercially prepared apple juice was guaranteed to contain 35 mgs. of vitamin C per 100 ml., but is believed to have been originally fortified to carry 50 mgs. of vitamin C per 100 ml.

The juice was dosed to the animals on the basis of its original fortification. Post-mortem examination, however, revealed that clinical symptoms of scurvy (manifested by severe hemorrhage in the lower limbs, beading of the ribs, etc.), were evident in 8 of the 10 animals on the 0.5 mg. level of feeding. Of these 8, 4 had shown macroscopic signs of scurvy while the experiment was still in progress. Slight hemorrhage was also observed in the lower limbs of 4 of the 10 guinea pigs on the 1.0 mg. feeding level. At the 2.0 mg. level of feeding, however, the odontoblast response to the apple juice exceeds the response of the odontoblast cells to synthetic ascorbic acid. It is difficult to offer an explanation for this result, unless the factor postulated to be present in natural foodstuffs is present in apple juice to the extent that, at this feeding level, it more than compensates for a smaller amount of vitamin C.

The somewhat elevated odontoblast response to the 2.0 mg. dose of artificial orange juice is unaccountable, although it is interesting to observe that odontoblast response to the 0.5 mg. and 1.0 mg. levels of the artificial orange juice does not differ from that to the crystalline vitamin. This might be interpreted as further evidence for the existence in natural vitamin C sources of a factor

(or factors), not present in the synthetic vitamin, which acts either synergistically with or independently of vitamin C to enhance the value of the foodstuff in fostering the development of odontoblast cells.

The growth response to supplements of natural orange juice, artificial orange juice, fortified apple juice and crystalline ascorbic acid is graphically illustrated in Figure II, page 38. The elevated growth response to artificial orange juice is unexpected and, in the light of present knowledge, cannot be explained.

As measured by growth response, both natural orange juice and fortified apple juice appear to be inferior to crystalline ascorbic acid until they are fed at a level above 2.0 mg. daily, when they begin to exceed the synthetic vitamin in growth-promoting value. Through this observation, it might be inferred that when growth is the sole criterion of response, these supplements should be fed in larger volumes. The "increase in weight" method is then unsuited to the measurement of small differences or of low potency substances.

The chemical and "odontoblast" assays of the vitamin C potency of natural orange juice and artificial orange juice are in close agreement (Table 9, page 43). It will be noted, however, that the biologically determined upper limit of the potency range for fresh orange juice is higher than for

either artificial orange juice or apple juice, while the lower limits of all three are comparable. The "odontoblast" method has attributed to fortified apple juice a higher potency than that given to it by the chemical method. In the biological determination of the average potency of the apple juice, the odontoblast response to the 0.5 mg. level of intake was disregarded, due to the scorbutic condition of the animals in this low-intake group. The odontoblast response to the 1.0 mg. and 2.0 mg. intake levels is marked, and the determination of the potency from these two results attributes to the apple juice a potency value higher than that obtained by chemical assay.

The "growth" method charges the assay materials with vitamin C potencies that do not agree with those obtained by the "odontoblast" and chemical methods of assay.

CONCLUSIONS

Using the "odontoblast" method of vitamin C bioassay, the development attained by the odontoblast cell is not different whether guinea pigs are penned and fed individually or in groups of two. Guinea pigs may also be assigned to assay at either a fixed initial age or a fixed initial weight, odontoblast development remaining unaffected.

There is evidence that natural sources of vitamin C are superior to synthetic ascorbic acid in promoting the development of the odontoblast cell. In determining the vitamin C potencies of natural foods by the "odontoblast" method, however, there is a need to assay these substances at two levels intermediate between 0.5 mg. and 2.0 mg.

The "odontoblast" assay of vitamin C affords greater experimental precision that the "increase in weight" method of vitamin C assay. In the assay work reported in this thesis, the "odontoblast" method is 300% more accurate than the "increase in weight" method.

Whereas all "tooth" methods have the disadvantage of requiring lengthy histological procedures, the "odontoblast" method has an advantage over other "tooth" methods in being fully objective.

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APPENDIX I

TRIAL I ALLOTMENT PLAN

Level of Ascorbic Acid (mg)	Sex	Mean Height Attained by the Odontoblast Cells of Each Animal (microns)	
		Control Ascorbic Acid	Fresh Orange Juice
0.5	♂	24 32 27 26 26	35 42 38 30 35
	♀	30 31 31 25 27	30 28 29 36 30
1.0	♂	36 36 35 37 42	40 43 44 46 40
	♀	37 34 35 39 36	45 46 41 35 47
2.0	♂	44 39 54 45 46	45 46 42 45 45
	♀	52 47 42 43 49	51 46 47 49 43

TRIAL I "Odontoblast" Method

Analysis of Variance

Source of Variance	D/F	Mean Squares
All Causes	59	
Between Subgroups	11	
Between Vitamin Levels	2	1210.7*
Between Sex	1	0.2
Between Assay Materials	1	211.7*
Interaction	7	29.2
Error	48	12.5

* Significance exceeds the 5% level.

TRIAL II ALLOTMENT PLAN

Level of Ascorbic Acid (mg)	Feeding Management	Sex	Mean Height Attained by the Odontoblast Cells of Each Animal (microns) Control Ascorbic Acid	Fresh Orange Juice	Artificial Orange Juice	Canned Fortified Apple Juice
0.5	Group Penning	♂	25	25	23	19
		♂	22	16	19	15
		♀	26	26	25	17
		♀	22	28	21	17
	Individual Penning	♂	24	28	28	20
		♂	18	25	20	14
		♂	25	24	23	17
		♀	20	22	25	15
		♀	23	23	20	20
		♀	15	22	20	16
1.0	Group Penning	♂	21	29	27	29
		♂	29	30	26	27
		♀	32	32	29	29
		♀	32	27	30	27
	Individual Penning	♂	29	22	26	31
		♂	24	30	32	31
		♂	29	36	29	32
		♀	30	30	30	28
		♀	37	35	32	21
		♀	30	30	29	17
2.0	Group Penning	♂	32	40	30	39
		♂	34	35	34	34
		♀	33	32	41	31
		♀	37	30	39	45
	Individual Penning	♂	44	34	35	36
		♂	33	35	35	32
		♂	33	33	38	36
		♀	32	33	38	37
		♀	30	38	32	39
		♀	36	37	38	39
4.0	Group Penning	♂	40	38	38	34
		♂	35	36	38	35
		♀	32	37	35	37
		♀	32	34	35	35
	Individual Penning	♂	36	42	35	36
		♂	36	34	34	34
		♂	38	39	33	38
		♀	35	32	32	39
		♀	36	34	42	32
			36	32	38	39

TRIAL II "Odontoblast" Method

Analysis of Variance

Source of Variance	D/F	Mean Squares
All Causes	159	
Between Subgroups	63	
Between Vitamin Levels	3	1840.4*
Between Sex	1	0.2
Between Assay Materials	3	31.0*
Individual and Group Penning	1	0.7
Interaction	55	16.9
Error	96	10.3

* Significance exceeds the 5% level.

APPENDIX II

THE PREPARATION OF GUINEA PIG INCISORS FOR MICROSCOPICAL EXAMINATION.

A method outlined by F.W. Gairns[1] for bringing tooth specimens to paraffin through nitric acid decalcification, has proved to be an improvement over the formic acid-sodium citrate decalcification technique formerly employed in this work.

Gairns' treatment has little effect of shrinkage on the pulp so that it and the odontoblast border lie in an undistorted relationship. A more satisfactory decalcification is accomplished in less time than that required by the formic acid-sodium citrate combination.

There follows a description of the histological and micrometric methods used in this laboratory in connection with the bioassay of vitamin C by the odontoblast method.

At the conclusion of the test period the animals are chloroformed. The lower jaw of each guinea pig is removed and divided by making a vertical incision between

- -

1. F.W. Gairns, Stain Tech., 19, 4 (1944).

the incisors. The exposed portion of the incisor and that portion of the mandible extending beyond the molars, are clipped to allow the fixing agent to reach the pulp.

Following a minimum fixation period of 48 hours in 10% formalin, the teeth are washed in 70% alcohol for 24 hours.

Decalcification is then carried out in 10% nitric acid, changing the acid every second day.

In 36-48 hours the unwanted molar and 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 easily pierced. Complete decalcification is usually accomplished in 3-4 days.

After rinsing in one or two 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 12-24 hours using a washing bobber[2] and are prepared for embedding in the following steps:

- -

2. F.W. Gairns, Stain Tech., 17, 131 (1942).

10% alcohol : 9 a.m. - 12 noon

20% alcohol : 12 noon - 5 p.m.

40% alcohol : overnight

60% alcohol : 9 a.m. - 12 noon

80% alcohol : 12 noon - 5 p.m.

Phenol added to these alcohols up to 6% imparts an elasticity to the tissues making possible the cutting of thinner sections[3].

Absolute alcohol: overnight - 10:30 a.m., changing the alcohol at 9 a.m.

Cedarwood oil : 10:30 a.m. - 12 noon

52° C. paraffin : 12 noon - 2:30 p.m.

60° C. paraffin : 2:30 p.m. - 4:30 p.m., changing once.

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

The guinea pig tooth exhibits persistent growth. In one tooth, at any one time, odontoblasts occur as embryonic, mature or senile cells. In transverse section, a normal tooth will show odontoblasts (and other cells) in either an immature, mature or degenerate condition depending on the level at which

- -

3. A.C. Lendrum, J. Path. and Bact., 60, 2, 416, (1935).

the sections are taken. It thus becomes necessary to examine longitudinal sections in which the entire history of cell development may be traced. The odontoblasts which are at the peak of development may then be selected for measurement.

Cutting

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

Sections are 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, 4-6 sections of 8-10 microns in thickness are selected for microscopical examination.

Staining

Erhlich's acid haematoxylin and the counterstain, eosin, are used in ordinary progressive staining technique.

Measurements

Measurements of odontoblast height are made under a 400 x lens by means of an ocular micrometer and are subsequently converted to microns. The odontoblast row of

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

Measurements are never made in the regions of the dental papilla or biting end where the odontoblasts are seen in their embryonic and senile states respectively.

Between these two well-defined regions lies the central area of maximum (mature) odontoblast development. In this area cell groups of the same height are chosen for measurement.

(Plate I)

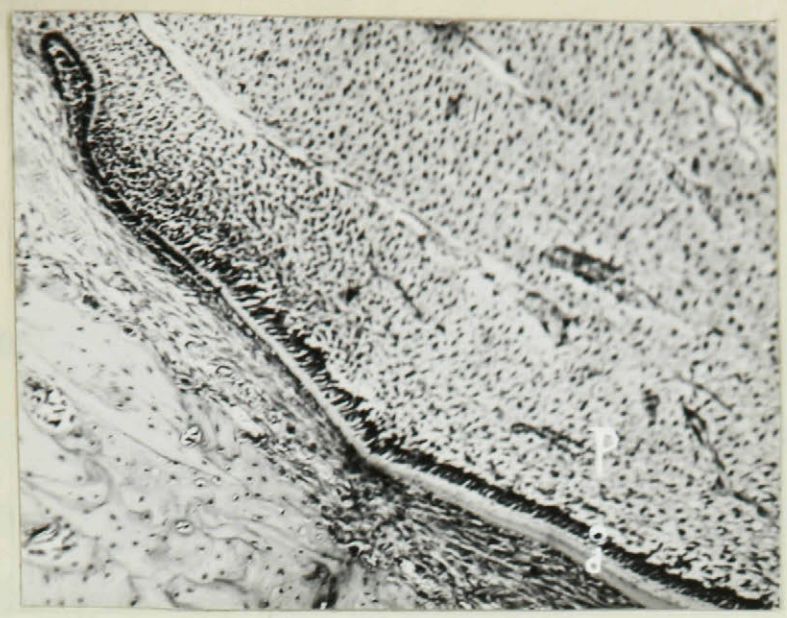


Fig.1 Formative end of incisor tooth showing embryonic odontoblasts.

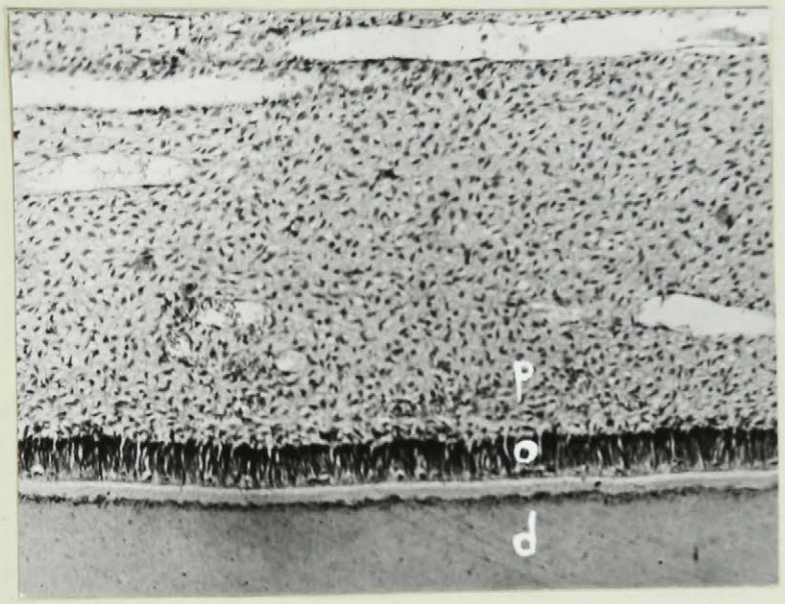


Fig.2 Mature odontoblast cells in area of reading

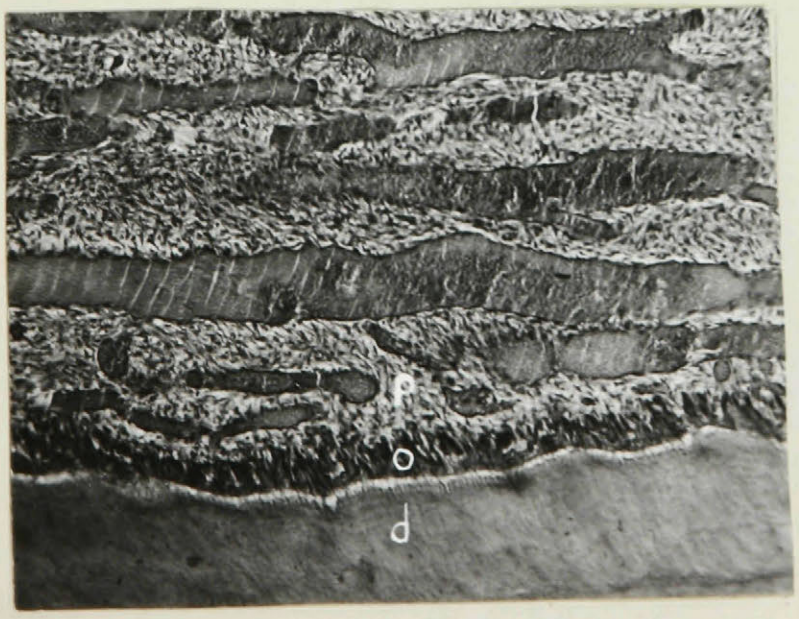


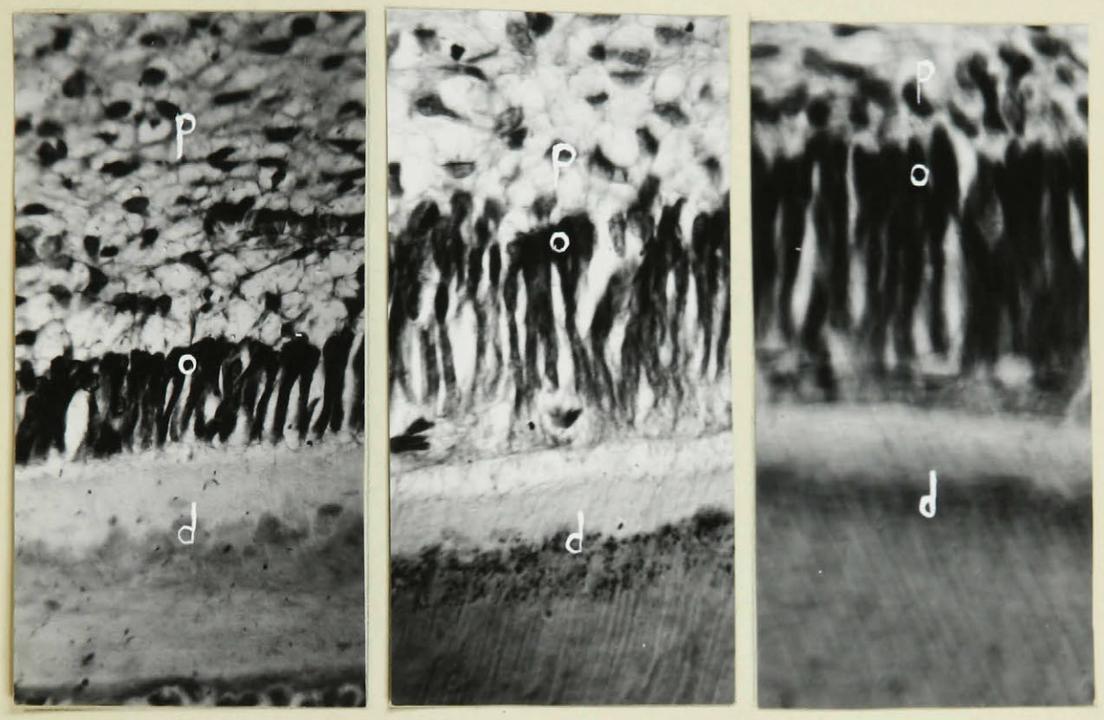
Fig.3 Incisal end of incisor tooth showing senile odontoblasts.

PLATE I. Photomicrographs showing stages of odontoblast development in the normal guinea pig incisor tooth.

(p) pulp (o) odontoblasts (d) dentine

When the guinea pig receives 0.5 mg. or more of vitamin C daily, the odontoblast boundaries from nucleus to basement membrane are clear-cut and cell height is easily measured.

(Plate II)



0.5 mg 1.0 mg 2.0 mg

PLATE II Photomicrographs (x 440) showing development of the odontoblast cells in guinea pig incisor teeth resulting from the daily intake of three different levels of vitamin C.

(p) pulp (o) odontoblasts (d) dentine

When the daily vitamin C intake of the guinea pig is as little as 0.25 mg., the odontoblasts are seen as an irregular row of abnormal cells, and a reliable estimate of odontoblast cell height is rarely possible.

(Plate III)

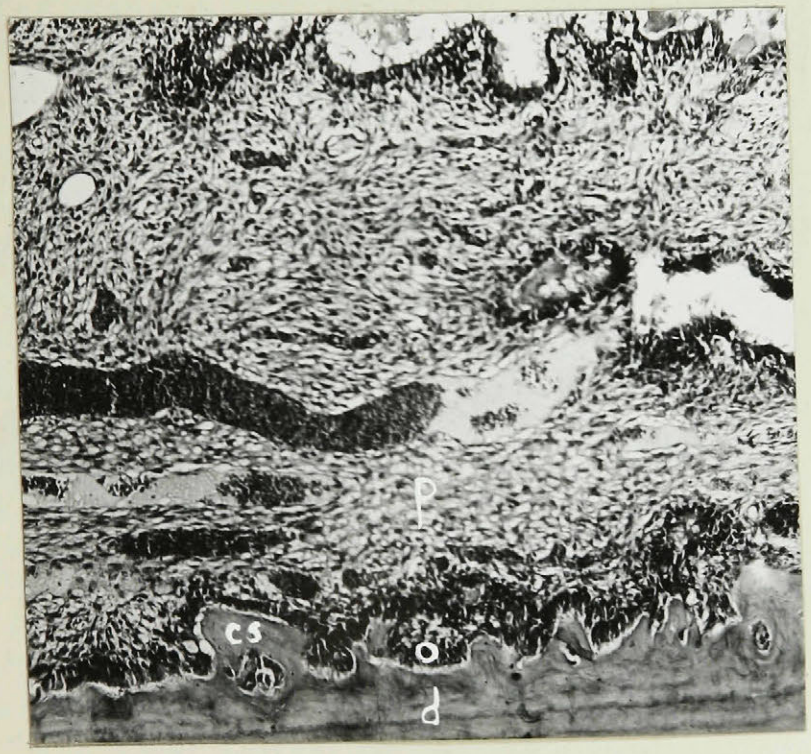


PLATE III Photomicrograph (x 100) showing irregular odontoblast row in a scorbutic tooth.

(p) disorganized pulp (cs) calcific scar tissue (o) degenerating odontoblasts (d) dentine.