

INCISOR TOOTH ASSAY OF VITAMIN C:
MICROMETRIC MEASUREMENT OF THE ODONTOBLAST CELLS

by

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A THESIS

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ABSTRACT

This thesis is a report of a vitamin C bio-assay using the maximum height to which the odontoblast cells develop in the incisor teeth of guinea pigs as the criterion for the state of scorbutus. Due to the removal of a considerable part of the subjective error present in other incisor tooth assay methods, highly reproducible results were obtained by this procedure. The accuracy of the odontoblast cell measurement method is shown to be far greater than the increase in weight criterion.

A detailed description of the preparation of the incisor teeth for microscopic examination and method of measuring the odontoblast cells is given.

A report of the post mortem lesions found is included.

INTRODUCTION

Interest in the bio-assay of vitamin C has increased during the past few years. The renewed research in this field has been a result of two factors: the increasing revelation of discrepancies in the chemical assay, coupled with the demand for a knowledge of the amount of available vitamin C in certain food-stuffs supplied to the Canadian armed services.

Growth response, post mortem lesions, and microscopic changes in the costochondral junctions and in the incisor teeth of guinea pigs have been the scorbutic criteria used to date. A great deal of variability in response and evaluation of that response to different amounts of vitamin C intake has been characteristic of these criteria. The changes occurring in the incisor teeth have been considered as the most reliable method of bio-assay so far developed. This procedure is also subject to much variation, mainly due to the differing personalities of the interpreters, even when they are evaluating the same tooth section.

It therefore seemed desirable to develop an impersonal method of evaluating histologically detectable scorbutus, with the elimination of as much of the

controllable variation as possible.

This thesis is a report of such an incisor tooth assay procedure. The two outstanding features of it are: (1) simplicity of interpretation with little requirement for histological or pathological knowledge; (2) reproducibility, by reduction of the variation in results between different laboratories by greatly minimizing the personal influence of the interpreter.

The method is based on the micrometric measurement of the odontoblast cells of the incisor teeth. It is well known that the odontoblast cells of scorbutic guinea pigs are subnormally developed to varying degrees, depending on the fraction of the optimum intake of vitamin C fed to the animal. Normal development of the cells is obtained when sufficient vitamin C is fed.

Thus, by the measurement of the odontoblast cells from guinea pigs on different levels of daily intake of ascorbic acid, it was thought that an accurate and reproducible method of evaluating the visible state of vitamin C nutrition of the guinea pig might be possible.

REVIEW OF LITERATURE

The first detailed report of the effect of the lack of vitamin C on the tooth structure of the guinea pig was that of Zilva and Wells (1919). Their description of fibroid degeneration of the pulp cavity tissue as it occurs during scurvy marked a transition of thought in regard to pathology. It was there that it was first considered that degeneration, which up to that time had been attributed to only inflammatory causes, could also be produced by dietary deficiency.

Since that time many experimental studies have been conducted for the purpose of further investigation into changes occurring in the teeth of guinea pigs subsisting on a scorbutic diet. Among the most prominent of these works are those published by Hojer (1924, 1926), Wolbach and Howe (1926), Goettsch (1928), Dalldorf and Zall (1930), Key and Elphick (1931), Fish and Harris (1935), Boyle (1938), Boyle, Bessey and Howe (1940).

The pathology of vitamin C deficiency in the teeth of guinea pigs has been extensively considered in these publications. For this reason and as the changes occurring in the odontoblast cells are the

primary consideration here, it is superfluous to review all the findings. For reference, an excellent treatise on the subject has been prepared by Dalldorf (1938).

It is accepted by all that the odontoblast cells are, as far as is ascertainable at present, the first cells in the body to exhibit histologically detectable changes due to vitamin C deficiency. This deficiency first causes a shortening and disorganization of the odontoblast cells. As the deficiency state becomes more intense, the disruption of the normal state becomes greater. Finally, during complete scorbutus, in place of tall, columnar, uniformly developed cells, there appears an extremely disorganized group of very short cells that may even become indistinguishable from the fibroblasts of the pulp cavity.

Previous investigators (Hojer, 1924, 1926; Goettsch, 1928; Key and Elphick, 1931) have used this pathological manifestation along with characteristic scorbutic changes in the pulp and dentine as their criteria for the degree of scorbutus present in the animal under question.

Hojer (1924, 1926) was the first to advance

an incisor tooth assay method. He described ten stages in the development of scurvy.

Goettsch (1928), due to the great variability between individuals, found it difficult to divide scurvy into so many fractions of complete protection. She compared the antiscorbutic potency of two substances by the minimum amount of each required to produce complete protection from scorbutic changes in the teeth. By this method, no use was made of results given by animals receiving less than the minimum fully protective dose. These suboptimum doses could not be evaluated unless it was assumed that the appearance of the teeth is entirely dependent upon the amount of vitamin C fed to that animal. From the results of the work of Goettsch, it appeared that this assumption was true. With close agreement between the dose of an antiscorbutic and the degree of tooth changes accepted, the amount of a substance of unknown potency required to produce full protection from scurvy could be calculated, provided a standard was available for comparison.

Key and Elphick (1931), following up this theory, partitioned the scorbutic changes in the

incisor teeth into five groups, ranging from 0 or no protection to 4 or full protection from scorbutus. By the use of this scale and employing orange or lemon juice as the antiscorbutic, they were able to construct a response curve shown as a straight line.

Thus by comparing the tooth response of guinea pigs receiving a fixed amount of an unknown antiscorbutic with the standard response curve, the potency of the unknown in relation to that of orange juice could be determined.

Crystalline ascorbic acid has to-day replaced orange or lemon juices as the standard.

Dann and Cowgill (1935), Harris and Olliver (1942), and Harris and Ray (1933) are some of the recent investigators that have used the incisor tooth assay method, based on the publications of Hojer, and Key and Elphick.

EXPERIMENTAL PROCEDURE

The data presented in this thesis were derived from a total of 192 observations.

Guinea Pigs.

The young guinea pigs used were born and raised to weaning in our own laboratory. All of the mothers of these young received the same basal diet. Some groups of the mothers differed in that they received varying dietary supplements during pregnancy and lactation. No young guinea pigs, however, were placed on test unless normal as to birth weight and gains to weaning. The average normal birth weight was 109.5 grams; the mean daily gain to weaning was 8.4 grams.

The guinea pigs were placed on test between the ages of three to four weeks at which time they averaged 311 grams and 285 grams in weight for the males and females respectively, with a combined standard deviation of ± 47.4 grams. They were kept throughout the experimental period of eight weeks in individual, all-metal cages, with wire floors. The basal diet was supplied in individual metal containers to each animal.

Fresh water, renewed at least once daily, was made available at all times by the use of inverted glass bottles fitted with rubber stoppers and bent glass tubes.

Basal Diet.

The basal diet available to the young and adults, at all times, consisted of the following ingredients:

15%	ground oats
10%	ground wheat
25%	dried beet pulp
10%	linseed oilmeal
15%	skimmilk powder
5%	fishmeal
5%	wheat germ meal
15%	dried brewers yeast
4%	bone char
0.5%	salt (NaCl) iodized at 0.1% KI
0.5%	cod liver oil (400 D - 2000 A)

This mixture was pressed into small cylindrical pellets of about 3/16 inches in length and 1/8 inches in diameter.

Weekly feed consumption and live weight records were kept.

The plan of allotment for both experiments is shown in Table I.

Allotment Plan for Guinea Pigs.

	Sex	Vitamin C		Daily Dose of Ascorbic Acid in Milligrams					
		Vit. E	Vit. A	0.25	0.5	1.0	2.0	4.0	8.0
Experiment 139 - 1943	Males	3.0 mgm./day	425 1.u./day	562* 413	439 422	388 490	390 446	470 412	401 386
			N11	443 403	395 393	411 448	436 478	408 391	561 387
		N11	425 1.u./day	399 457	421 417	440 392	464 402	404 471	460 419
			N11	438 559	444 424	414 396	409 445	482 418	405 449
	Females	3.0 mgm./day	425 1.u./day	585 406	431 455	486 452	475 428	430 539	461 410
			N11	450 434	389 453	432 415	487 491	456 469	433 459
		N11	425 1.u./day	427 394	463 451	465 565	441 473	447 442	477 480
			N11	416 468	462 472	423 437	435 467	476 553	400 535
Experiment 139A - 1943	Males	3.0 mgm./day	425 1.u./day	700 628	657 667	647 633	646 691	674 653	622 635
			N11	683 654	643 618	720 686	655 698	629 641	665 663
		N11	425 1.u./day	638 693	666 669	626 662	678 651	661 709	705 692
			N11	630 699	679 672	637 639	623 694	690 668	624 701
	Females	3.0 mgm./day	425 1.u./day	702 642	609 707	714 688	708 616	611 703	723 617
			N11	673 631	632 687	606 614	710 711	682 656	684 658
		N11	425 1.u./day	625 621	685 675	671 696	649 695	697 627	636 689
			N11	619 652	660 640	620 648	634 670	664 704	650 706

*Animal identification number.

Table I.

Vitamin C Supplement.

The desired amounts of ascorbic acid were added to a sufficient quantity of a mixture of equal parts of cornmeal and cornstarch, so that 100 milligrams of the total mixture contained the required daily dose of ascorbic acid. The supplement for each lot was kept in a brown, screw-topped bottle. The 100-milligram daily dose of total supplement compound was given orally by means of a small glass tube with metal plunger, graduated to deliver the required amount.

Special Supplements.

Vitamin E.

Due to the results obtained from previous experiments in which muscle dystrophy of the type produced by vitamin E deficiency occurred, it was decided to fortify the diet with additional vitamin E, as alpha-tocopherol. This deficiency was present in spite of the fact that the diet contained 25% natural grains plus 5% wheat germ meal. According to Cummings and Mattill (1930), the destruction of the vitamin E may be explained through the oxidizing action of the unsaturated fatty acids in the cod liver oil and linseed

oilmeal in the diet.

The vitamin E (alpha-tocopherol)⁽¹⁾ was dissolved in corn oil in such concentration that 0.2 c.c. of the oil, fed orally on odd-numbered calendar days, alternating with the vitamin A, provided an equivalent of 3.0 milligrams of vitamin E per day.

Vitamin A.

As vitamin A is also susceptible to oxidation, but to a much less extent than vitamin E, an additional amount of this vitamin (carotene in oil preparation)⁽²⁾ equivalent to 425 I.U. per day, was fed on even-numbered days, alternating with the vitamin E.

Vitamin D.

Vitamin D, as Delsterol⁽³⁾, was incorporated in the vitamin C supplement mixture fed to all animals, in a quantity calculated to provide 42.5 I.U. per day.

(1) Supplied by Merck and Co. Limited, Montreal, P.Q.

(2) Provatol, 250,000 U.S.P. units per gram, supplied by Charles E. Frosst and Co., Montreal, P.Q.

(3) Delsterol, Canadian Industries Limited, 2000 I.U. of vitamin D₃ per gram.

Post Mortem and Histological Procedure.

Upon the death of an animal or at the conclusion of the eight weeks test period, an autopsy was performed. The post mortem lesions found in any of the tissues, whether considered due to the lack of vitamin C or not, were recorded. The two rami from the lower jaw of each animal were immediately placed in 10% formalin.

Decalcification of Teeth.

Following fixation of at least three days duration, the lower jaw was placed in a decalcifying solution. The decalcifying fluid used consisted of equal parts, by volume, of a 40% solution of formic acid and a 20% solution, by weight, of sodium citrate. The solution, 50 c.c. per tooth, was renewed twice the first day and once each subsequent day. About the second day, it was possible to discard the molar teeth by cutting them away from the incisor tooth, thus reducing the time required for the process. Complete decalcification of the teeth was accomplished in five days; four days for those teeth from animals in the 0.25 milligrams of ascorbic acid per day group.

Imbedding.

The decalcified teeth were then rinsed in 70% alcohol for 24 hours. The teeth were prepared for imbedding in wax in the following manner:

80% Alcohol	- 1 hour
95% Alcohol	- 2 hours
Amyl Acetate I	- 1 1/2 hours
Amyl Acetate II	- 1 1/2 hours
Wax I*	- 1 hour
Wax II	- 1 1/2 hours
Wax III	- 1 1/2 hours
Imbed	

Sectioning.

Longitudinal sections should be cut so as to include as much as possible of the pulp cavity, extending from the dental papilla toward the crown of the tooth. This is especially desirable in those teeth from guinea pigs receiving upwards of 1.0 milligram of ascorbic acid per day, as the development of the odontoblasts at these levels increases for some distance from the apex of the tooth.

*Fisher's Tissuemat Wax - 56-58° C. melting point.

Orientation of the imbedded tooth to the microtome blade should be such that as much as possible of the pulp cavity is revealed at the time the blade cuts into it. Sectioning is continued until the blade is perpendicular to the lingual and buccal surfaces of the tooth. This point is recognized by the disappearance of a thick white band bordering the pulp cavity, representing an oblique cut through the dentine. The dentine then becomes a similar colour to that of the surrounding tissues. The first section is taken at this point and from here on, four to six sections at 40 to 48 micron intervals are saved for examination. This procedure was adopted so as to ensure that one of the sections will be representative of that tooth.

Following examination of sections prepared in the above manner, it is now proposed that six sections taken at 56 micron intervals would give greater assurance of obtaining at least one section that is truly representative of that tooth.

Staining.

Ehrlich's acid-hematoxylin was used with eosin (saturated solution in 95% alcohol) as the counter stain.

The staining times are indicated below:

Xylol I	2 minutes
Xylol II	2 "
Alcohol 95%	2 "
Alcohol 95%	2 "
Alcohol 80%	2 "
Alcohol 70%	2 "
Alcohol 50%	2 "
Sodium thiosulphate (1 gm. in 400 c.c. of 50% alcohol).	2 "
Running water	3-5 "
Hematoxylin	45-60 seconds
Acid alcohol (1 c.c. of HCl in 100 c.c. of 70% alcohol).	In and out
Lithium carbonate (saturated solution)	Until blue
Running water	3 minutes
Alcohol 50%	Wash
Alcohol 95%	Wash
Eosin	2 minutes
Alcohol 95%	In and out
Alcohol 95%	" " "
Alcohol 95%	" " "
Alcohol 100%	" " "

Alcohol 100%		In and out
Alcohol 100% + Xylol (50:50)		" " "
Xylol I		" " "
Xylol II		" " "
Xylol III		" " "

Canada Balsam mounting.

Measurement of Odontoblast Cells.

Measurement of the distance from the dentinal end to the nuclear end of the cells is made. Cell groups in which two or three cells are of the same height should be chosen for measurement, avoiding single cell readings as much as possible. Due to the irregularity of the cells in the levels below 1.0 milligram (Plates I, II and III), the avoidance of single cell measurements is not always practical.

The cell nuclei should be seen as a clear cut row of dark bodies. On the higher levels of ascorbic acid, this row should be straight (Plate IV), becoming more uneven as the daily dose of ascorbic acid decreases. The outline of each cell must be traceable from the nucleus to the basement membrane. The basement membrane should be seen as a clear cut line

(Plate IV). This is not always possible especially on the lower levels (Plates I and II); however, an accurate estimate of the boundaries of the cell is almost always obtainable.

Some of the teeth from animals on the lower levels have well developed, newly formed odontoblasts just above the dental papilla. Because of this fact, measurements of these cells were not made until they had decreased in size. This decrease in size is quite sudden and easily recognized. Readings were made under 400X lens, by means of an ocularmicrometer and subsequently converted to microns.

Five separate readings were made from each tooth. The average of these was considered as representative of the maximum height of the odontoblast cells of that animal.

A few teeth will be encountered in which the odontoblasts lie at an acute angle to the dentine (Plate III) rather than perpendicular. In these cases, the reading must be taken parallel to the long axis of the cell and not at right angles to the dentine.

EXPERIMENTAL RESULTS

Analysis of variance of the odontoblast cell measurements (Table II) revealed two highly significant differences: one between the levels of ascorbic acid fed, the other between the two experiments.

The first of these, the difference between the heights of the odontoblast cells from animals on different levels of ascorbic acid intake was very marked (Chart I). The necessary difference between the means of the various levels was 2.3 microns. The smallest difference between the means of the levels up to the 2.0 milligram level is 7.9 microns.

The highly significant difference between the experiments is graphically present in Chart II. The reason for this is explained later.

There was no significant difference between the heights of the odontoblast cells from male or female guinea pigs, nor from those receiving additional amounts of either vitamins A or E. Chart I depicts the odontoblast cell height curve for males and females. It will be noted that the cells of both sexes developed to exactly the same average height at the 2.0 milligram optimum requirement level. This finding is in direct

Table II.
Analysis of Variance Showing the Effect of Different Amounts of
Ascorbic Acid Intake on the Height of the Odontoblast Cells.

Sources of Error	D/F	S(x- $\bar{x}$) ²	σ^2	σ	F Values	
					Observed	Necessary
Total	191	6,789.41				
Between Sub Groups	95	6,408.98	67.46			
A vs. no A	1	16.45	16.45		3.29	3.97
E vs. no E	1	17.64	17.64		3.52	
Levels of C	5	5,717.97	1,143.59	33.82	228.49	2.34
Between sex	1	.91	.91			
Between tests	1	145.61	145.61		29.09	3.97
Interaction	86	510.40	5.93		1.18	1.44
Calculated values	20					
Remainder	76	380.43	5.005	2.24		

Necessary difference between levels of vitamin C = $\frac{2.24}{\sqrt{32}} \times \sqrt{2} \times 1.992 \times 2.04^* = 2.28$ microns.

*2.04 = factor for converting the ocularmicrometer scale to microns.

contrast to the marked discrepancy in the gains in weight of the two sexes, as shown in Chart III.

A graphical comparison of the response of body weight and odontoblast cells to increasing amounts of ascorbic acid is seen in Chart IV.

Chart V reveals the constant degree of difference between the measurements made by two technicians, independently.

The odontoblast cell and body weight increase response curves are shown in Charts VI and VII.

Representatives of the cell types encountered on the various levels of ascorbic acid are shown in Plates I to IV.

Post mortem lesions are reported in the Appendix.

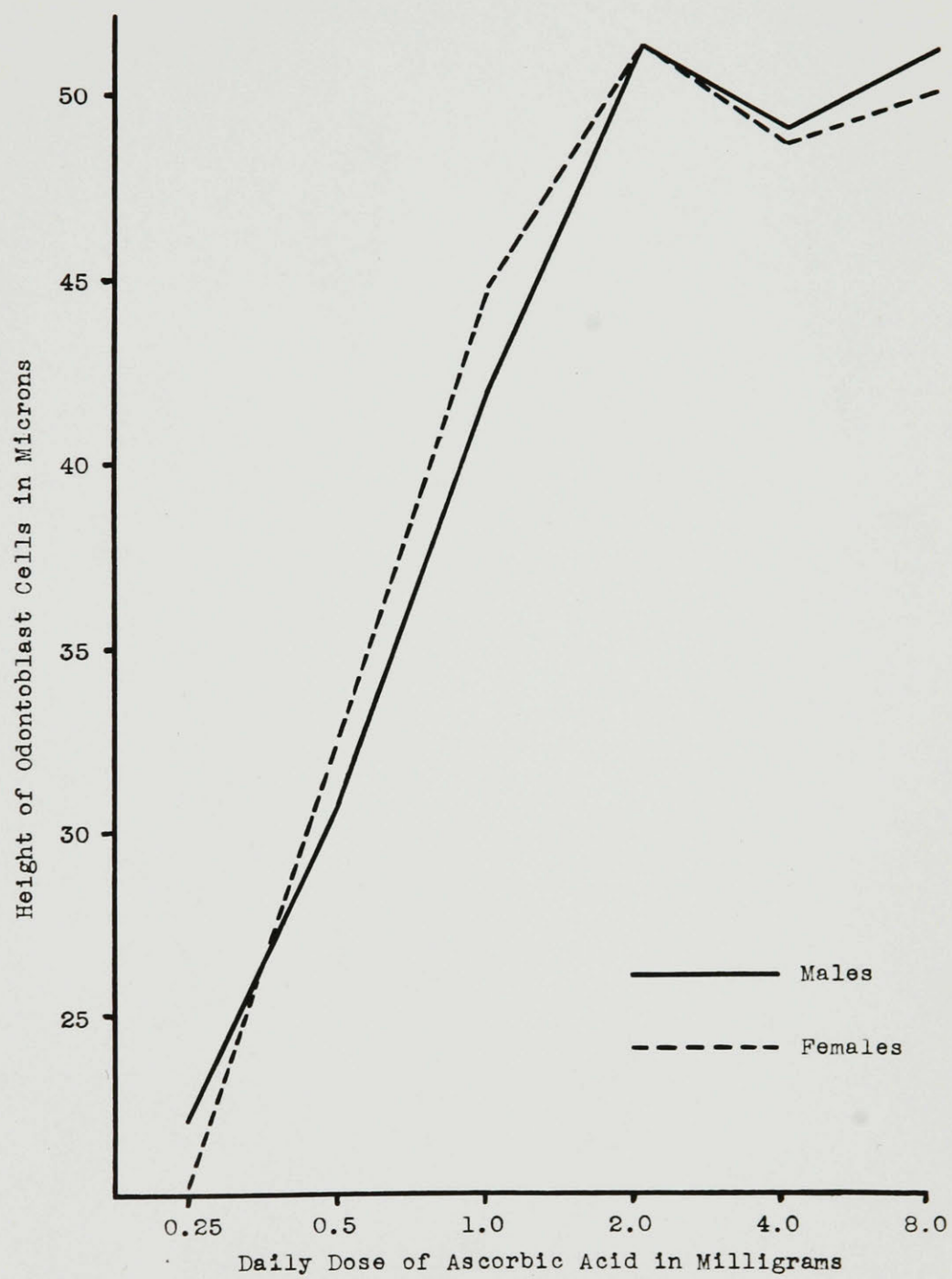


Chart I.

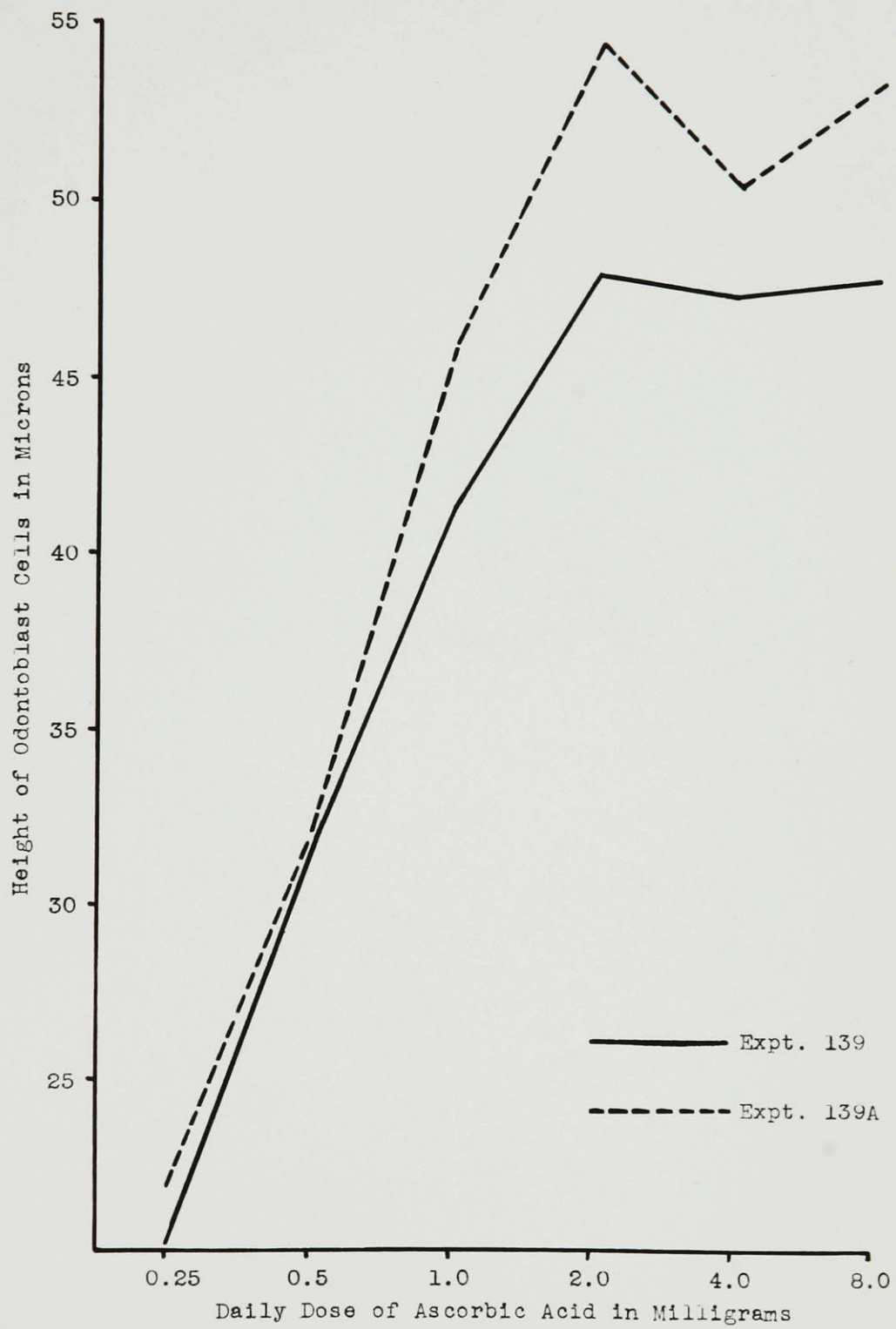


Chart II.

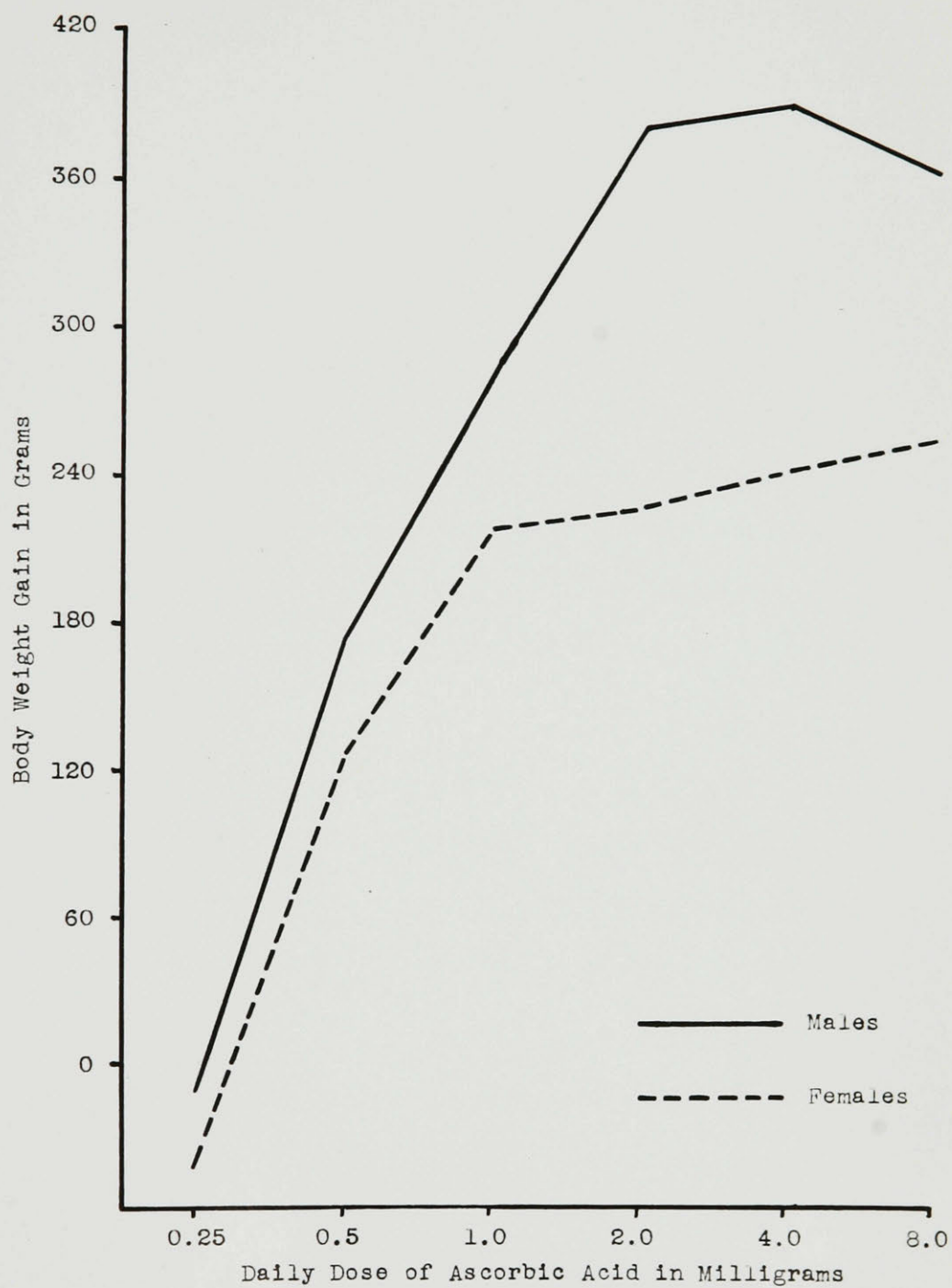


Chart III.

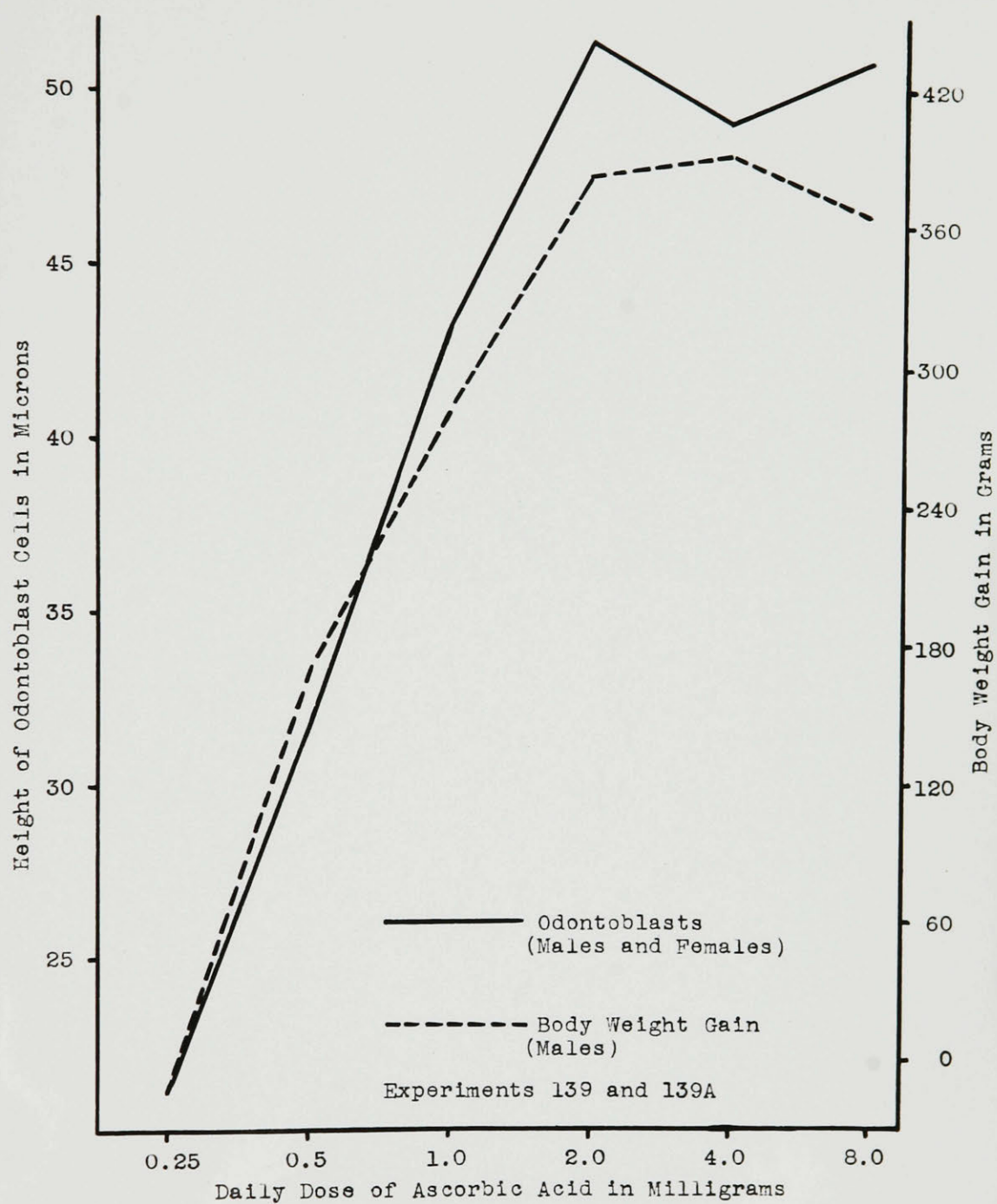


Chart IV.

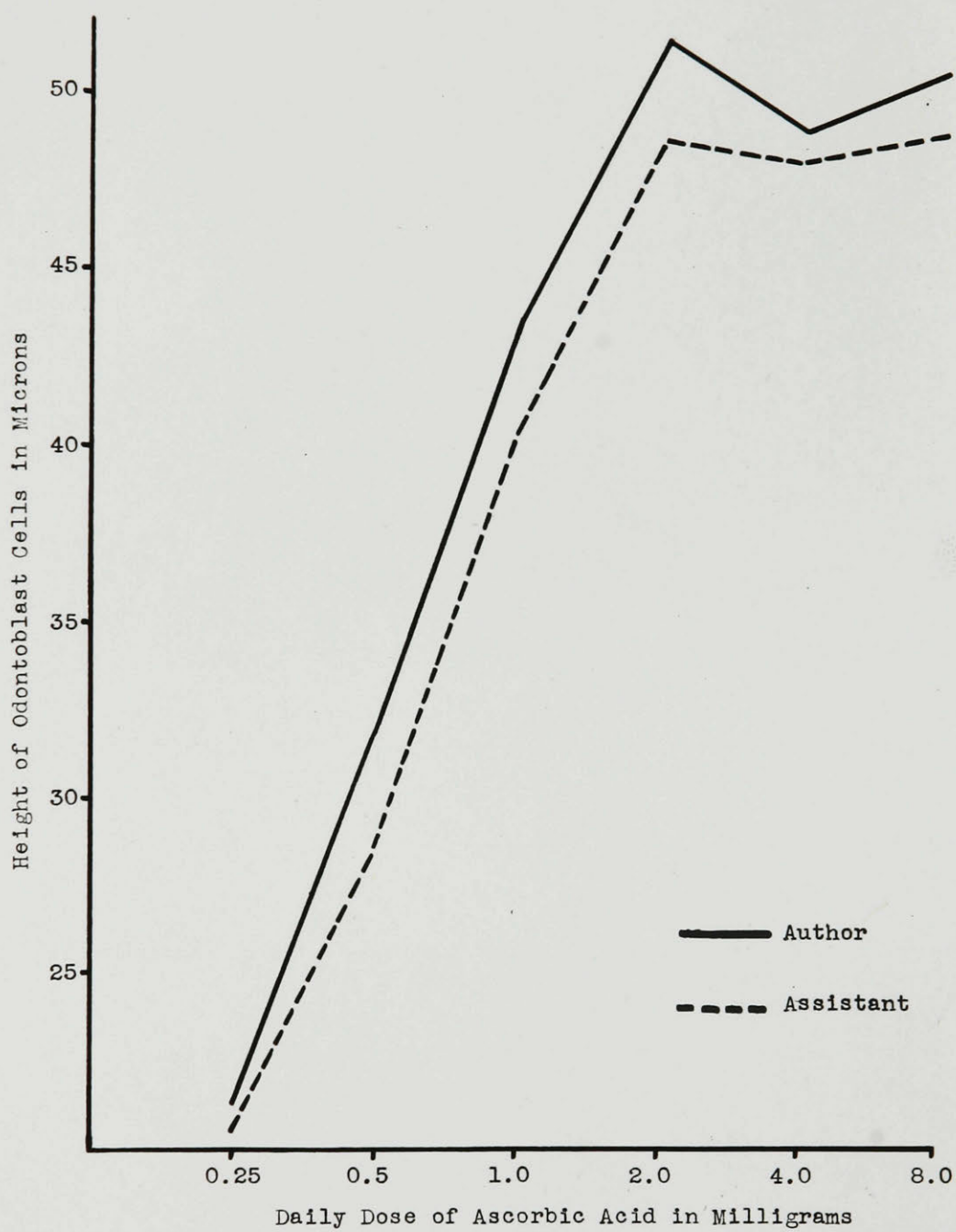


Chart V.

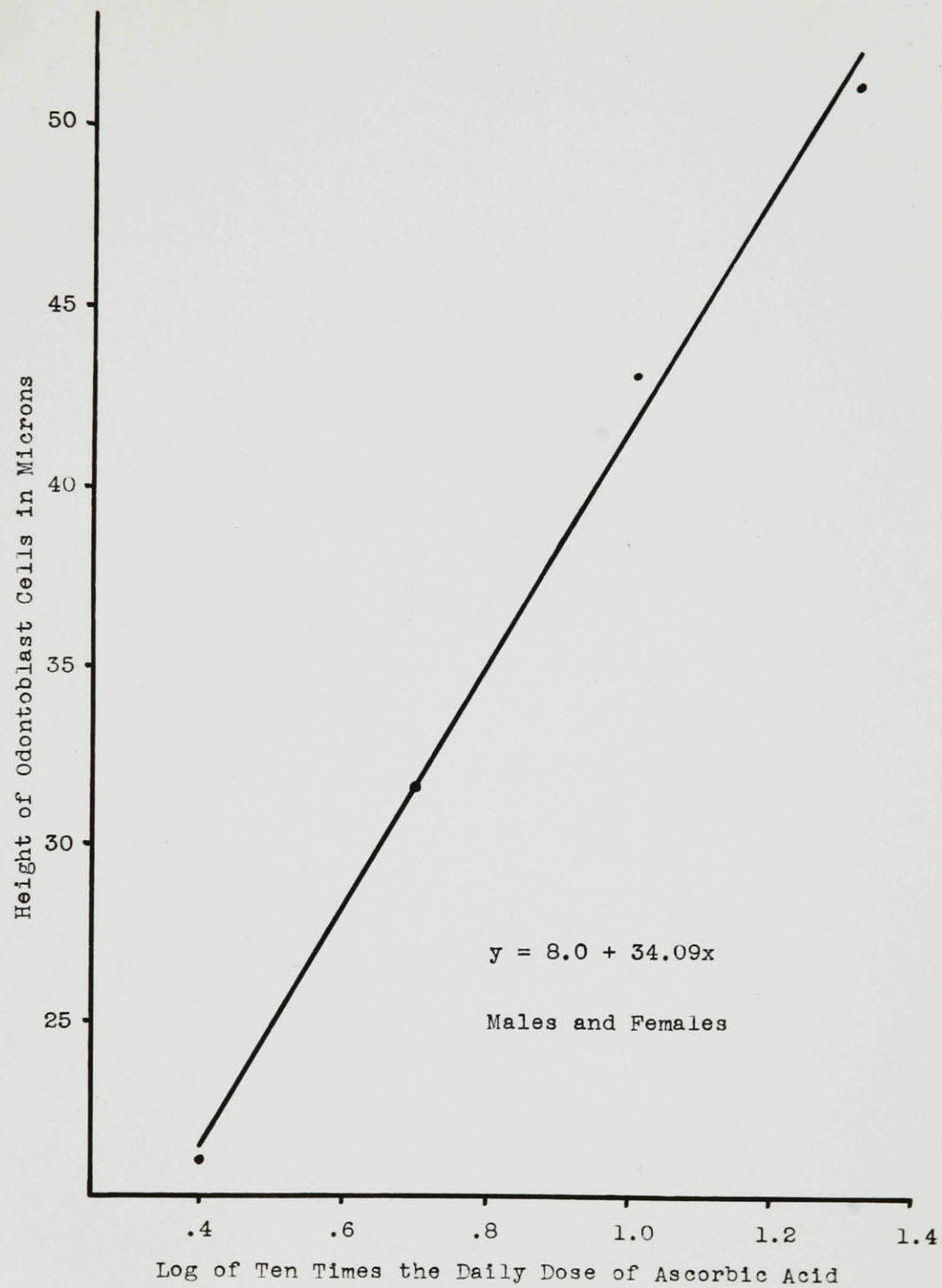


Chart VI.

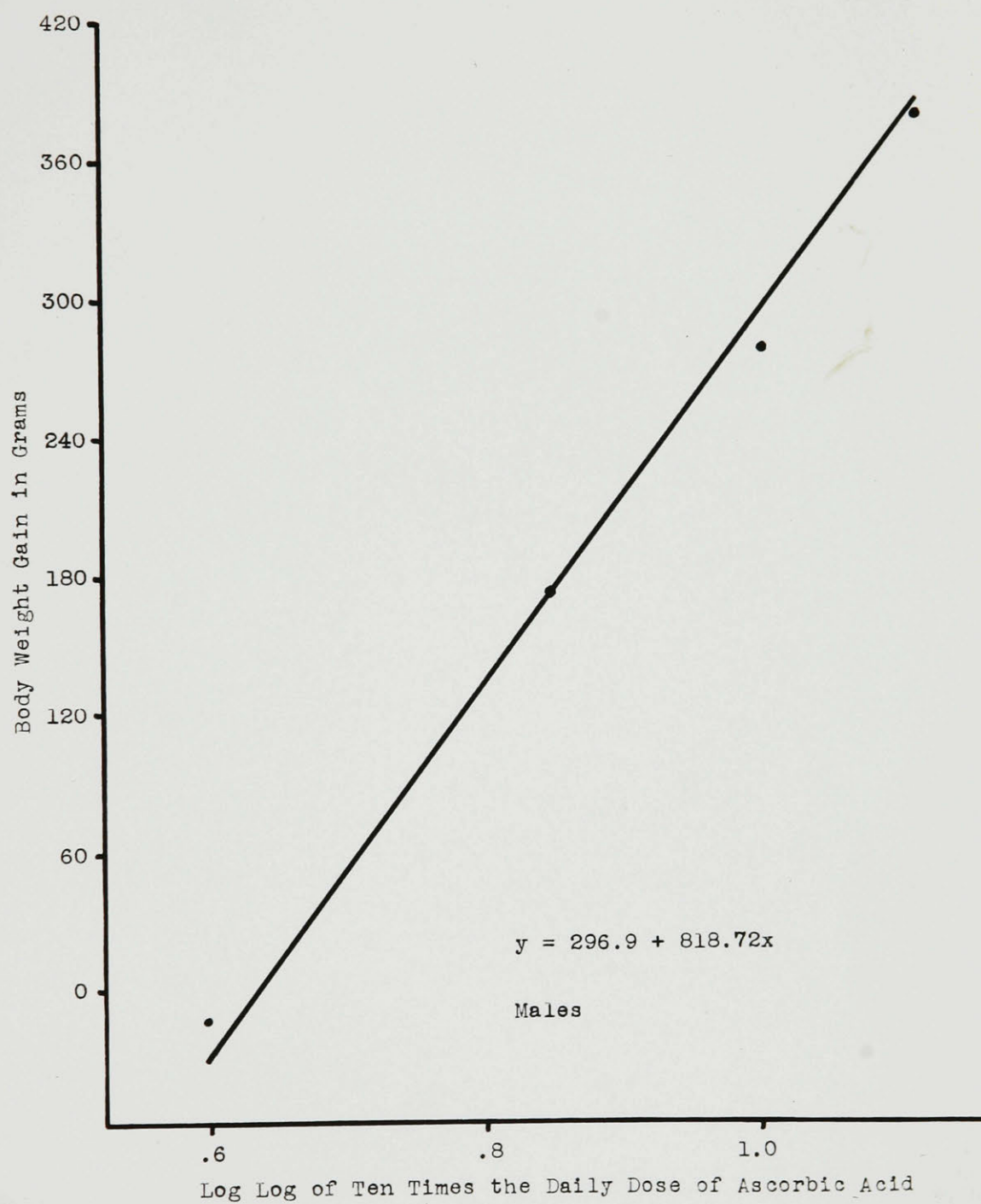


Chart VII.

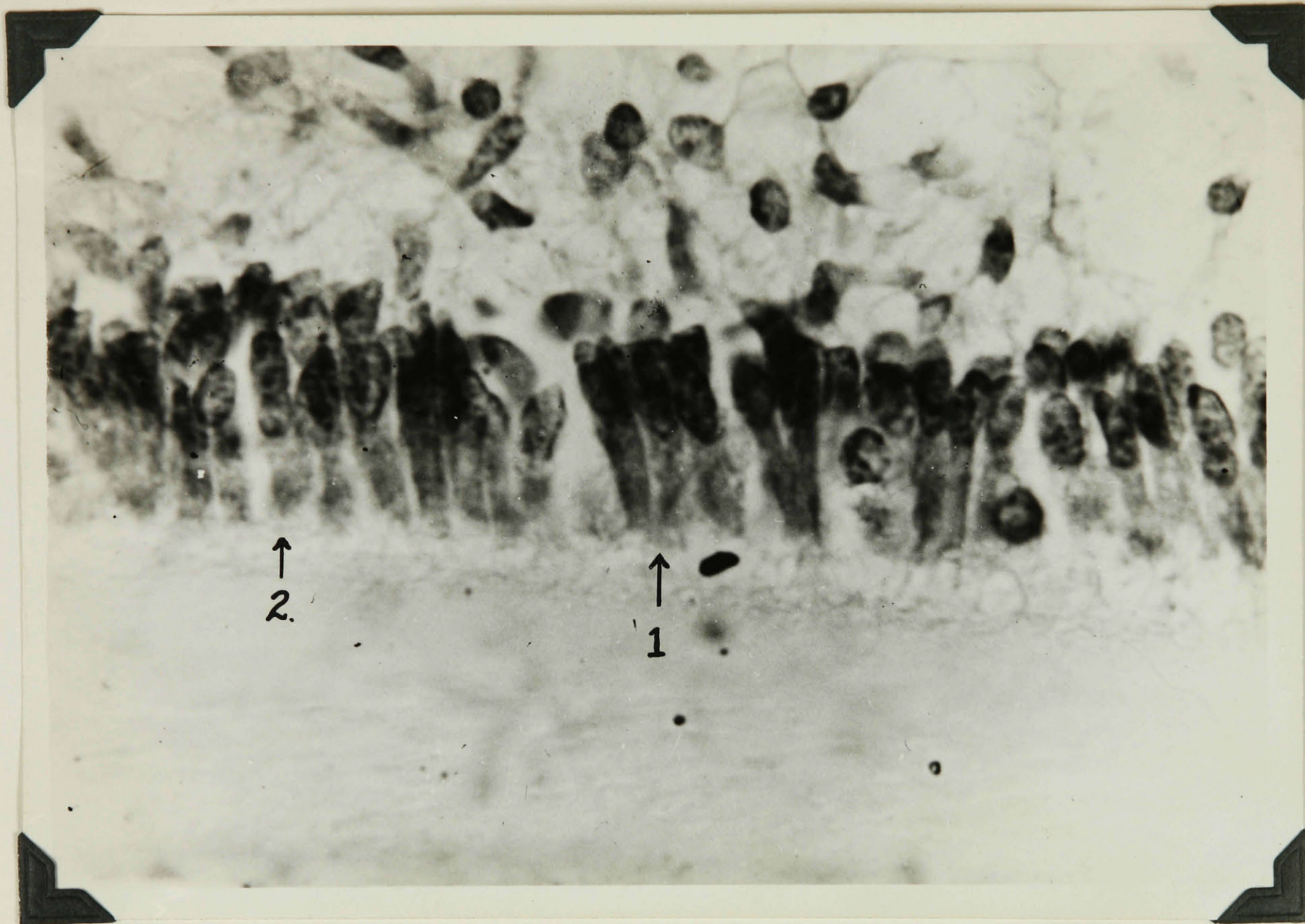


Plate I - 0.25 Milligrams Ascorbic Acid Daily

No. 1 arrow, pointing to the small group of odontoblast cells in the centre of the plate, indicates the position for an accurate reading of this field of odontoblast cells. No. 2 arrow, to the left of the centre, points to a region not suitable for taking measurements due to the tiered display of cell nuclei.

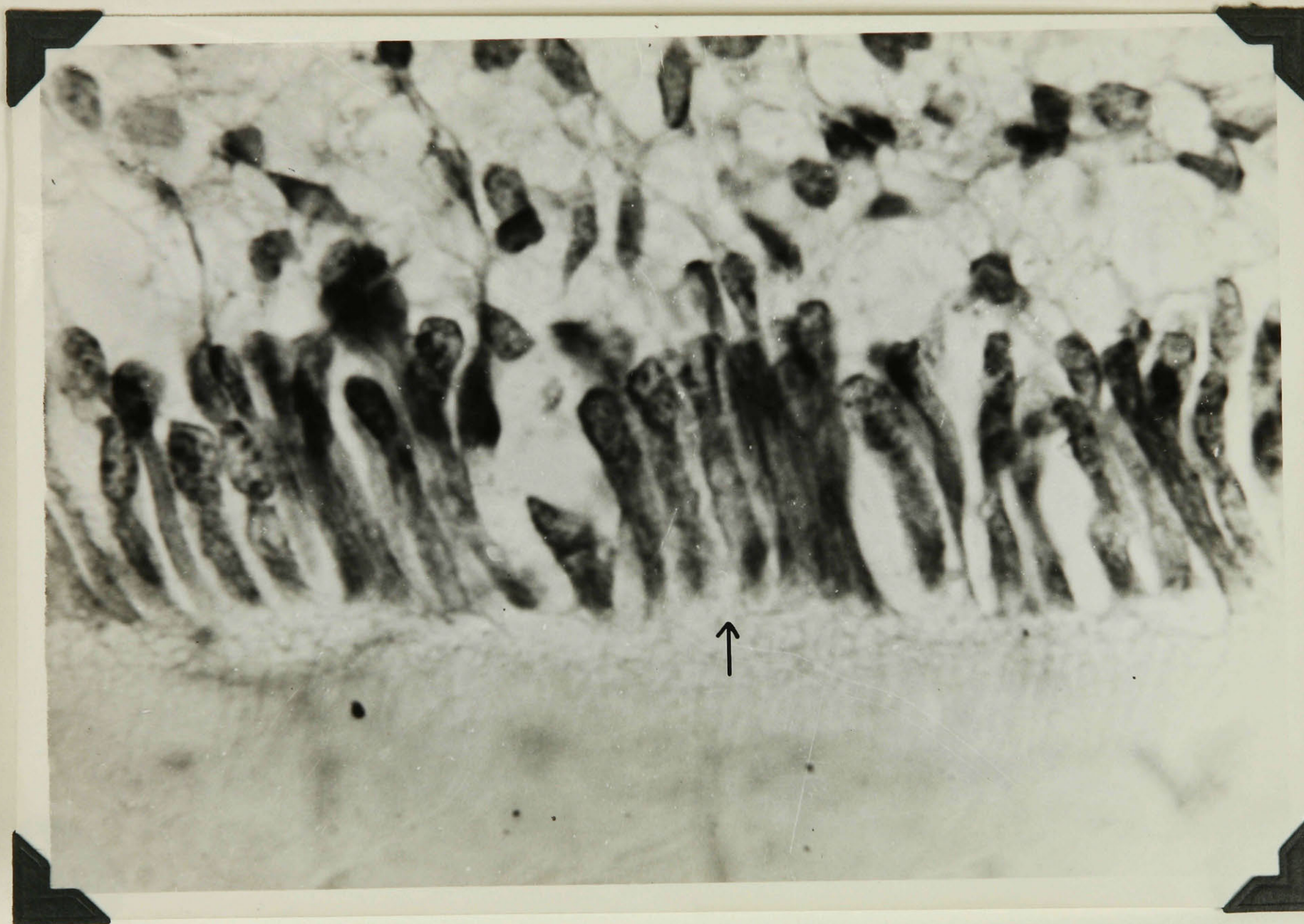


Plate II - 0.5 Milligrams Ascorbic Acid Daily

The arrow lies at the base of a measurable group of odontoblast cells.
The cells have elongated considerably, but are uneven in distribution
and wavy in outline.

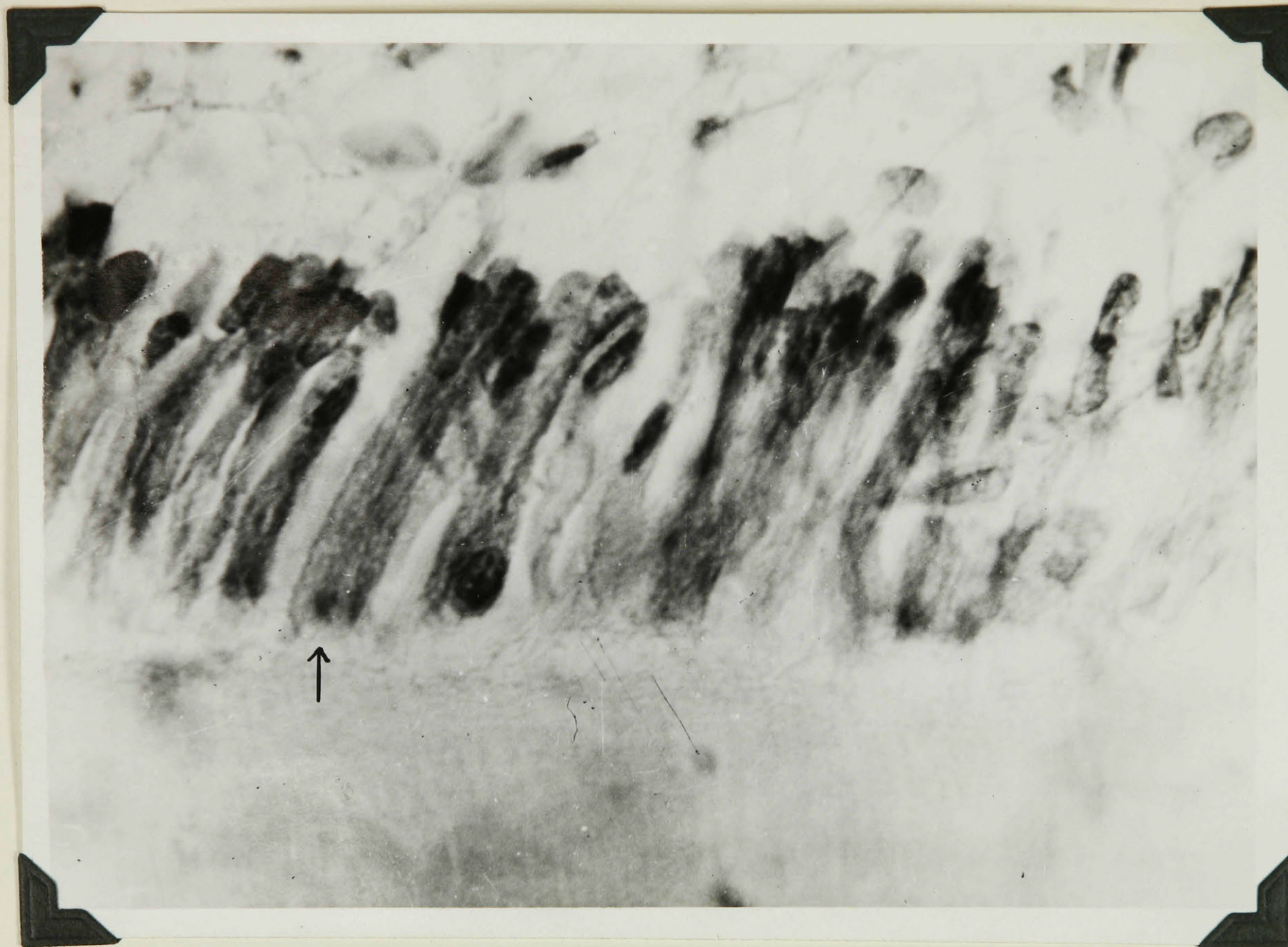


Plate III - 1.0 Milligrams Ascorbic Acid Daily

The cells have elongated still further, become straighter and more abundant. The cell nuclei form a straighter line bordering the pulp cavity. The arrow points to a measurable group of cells.

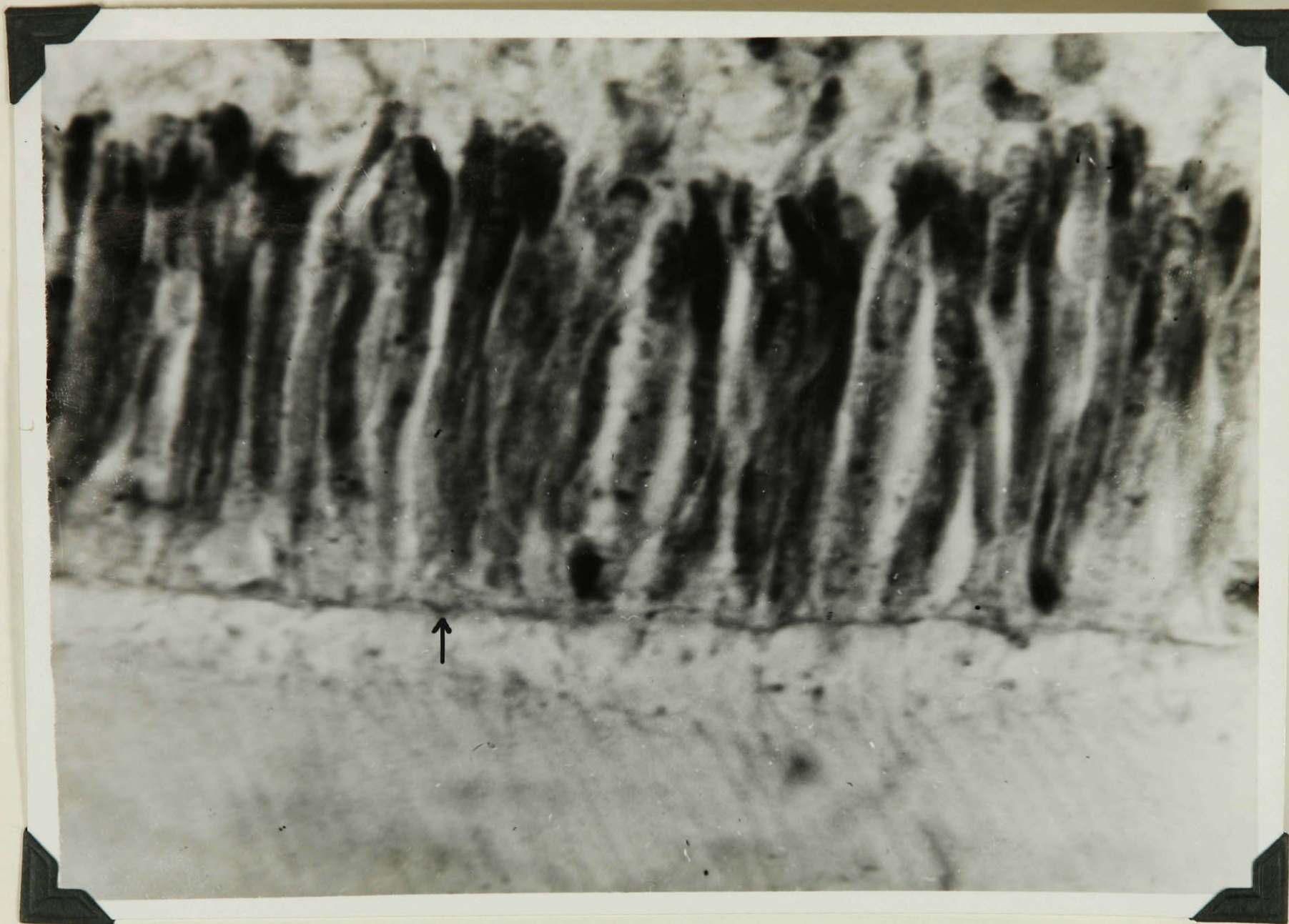


Plate IV - 2.0 Milligrams Ascorbic Acid Daily

This plate represents the maximum development of the odontoblast cells. They are uniformly tall and straight. The cells are traceable from the nuclear end to the well defined basement membrane.
The 4.0 and 8.0 milligram levels are comparable.

DISCUSSION

The incisor tooth assay methods as proposed by Hojer (1924, 1926) and Key and Elphick (1931), require a moderate knowledge of pathology, as well as the ability to establish a balance between the three main variables in the scorbutic tooth, the odontoblasts, dentine and Tome's canals, in evaluating the degree of scorbutus. The use of only one criterion in the incisor tooth, the odontoblast cells, and the evaluation of their degree of scorbutus by an average of five measurements of the most fully developed of these cells, removes the necessity for a knowledge of pathology and special training in establishing the state of scorbutus.

The basis for using only the odontoblast cells in determining the presence of scorbutus is sound. It has long been considered that the odontoblast cells of the guinea pig are the first cells of the body to be visibly affected by vitamin C deficiency.

Histologically detectable changes in the odontoblast cells occur as early as ten days after placing the animal on a scorbutic diet. It is upon this fact that when the curative growth method of assay is used, the feeding of the vitamin C supplement begins

on the eleventh day of subsistence on a scorbutic diet, just before the animal begins to lose weight. Thus the odontoblast cells are affected more quickly by lack of vitamin C than is the body weight. Body weight is affected by many factors other than vitamin C. The only other condition reported as affecting the odontoblast cells is that of vitamin A deficiency. Burn, Orten and Smith (1941) report that severe histological changes occur in the incisor teeth of the rat maintained for as much as a year on a vitamin A deficient diet. A deficiency as severe as was produced under their experimental conditions is not likely to be present in a scorbutic diet, but it serves as a warning that the vitamin A adequacy of the basal diet must be assured even when using only the odontoblast cells as the criterion of scurvy. This statement is further substantiated in this thesis when it is considered that, while there was no significant difference in the height of the odontoblast cells in those animals either receiving or not receiving additional vitamins A or E, there was a definite trend toward better development of the cells in those animals receiving additional supplements.

As may be seen in Chart II, there is a

considerable difference in the height of the cells in Experiment 139 as compared to those of Experiment 139A. This discrepancy between tests would be a serious objection to the procedure if it were not explainable. In Experiment 139, four sections were taken at 40 micron intervals beginning at the point as described under experimental procedure, whereas six sections were retained from the teeth in Experiment 139A. This was done in order to obtain sections that were more representative of the central region of the tooth as well as to include more of the pulp cavity toward the crown.

In making the measurements for Experiment 139A, the sixth, and less often the fifth sections were the only ones used, whereas the third and fourth sections were used in Experiment 139. This makes a difference of about 96 microns in the longitudinal plane from which readings were made in each test. In addition to this, a greater part of the pulp cavity toward the crown of the tooth was included in the second group of sections. This exposed more fully developed odontoblast cells in those teeth from animals on the higher levels where the more adequate intake of ascorbic acid allowed a greater development of the cells as they approached the crown of

the tooth. This is substantiated by referring to Chart II, where it will be seen that the greatest difference between tests is at the 1.0 and 2.0 milligram levels.

Statistical analysis reveals there is no significant difference in the height of the cells from male or female animals. This fact is of considerable importance. As may be seen in Chart III, the male guinea pigs grew much more quickly than did the females, so much so that the females are considered as unsatisfactory for vitamin C assays when the growth criterion is used. This necessitates the use of only male animals, which makes it far more difficult to obtain sufficient numbers of animals for a test unless a large stock colony is maintained. The use of the odontoblast cell criterion removes this difficulty, and is another indication of its specificity for vitamin C.

The simplicity of the procedure for measuring the odontoblast cells may be realized when it is considered that following two to three hours of instruction, any person with moderate intelligence and an understanding of the use of the microscope will be able to determine accurately the degree of histologically

detectable scorbutus in a section of a tooth in a very few minutes.

The objection to the other methods of tooth assay is that too great a discrepancy has existed between the values obtained by different persons from tooth sections of guinea pigs on comparable levels of vitamin C intake. An example of this is clearly shown in a comparison of the works of Harris and Ray (1933) and Key and Morgan (1933). Harris and Ray, using the incisor tooth assay procedure as proposed by Key and Elphick (1931), claimed that 1.0 milligram of ascorbic acid was equivalent to the activity of 15 I.U. Key and Morgan, using the same method, indicated a 7.4 I.U. activity for 1.0 milligram of ascorbic acid. Variations of this magnitude do not allow accurate comparison of results from different laboratories.

While it is true that in the experiments reported in this thesis, the means of the measurements of the two technicians are significantly different for all levels of ascorbic acid, one set, as has already been pointed out, is consistently lower than the other but the lines remain parallel throughout their course (Chart V). This discrepancy may be due to two factors.

Low values may be obtained by measuring those cells that are not the most fully developed odontoblast cells to be found in that tooth. This necessitates careful examination of a section to be sure that measurements are made of the most fully developed cells in that tooth.

Sections must be made, especially on the higher levels, so as to include all of the pulp cavity up to the point where the cells no longer continue to increase in height. High readings may result from including in the measurement, either an abnormally thick basement membrane due to faulty sectioning, or some of the undesired "tiered" cells. Some sections must be examined critically to determine the exact outline of the basement membrane of the cells. As the only stains used in this procedure have been hematoxylin and eosin, it might be possible to remove this source of error by staining with Mallory's connective tissue stain. This would bring the connective tissue and cell walls into greater relief.

The difference required for significance between the measurements of the two technicians, 0.5 microns, indicates the small amount of personal factor variability allowed. However, differences in corresponding values of the two operators ranged from

0.9 microns to 3.2 microns. This means that at no level were comparable results obtained. An indication of how easily a discrepancy of over 0.5 microns can be obtained may be realized when it is considered that one unit on the ocularmicrometer is equal to 2.04 microns, and cell measurements on the micrometer range from ten units on the low levels up to 24 units on the highest levels. These facts make it comparatively easy to obtain differences in readings of 2.0 or 3.0 microns.

The accuracy of the micrometric measurement of the odontoblast cells as a criterion for the degree of scorbutus in the guinea pig as compared to the growth method used in this thesis is very much in favour of the former.

The percentage error for the odontoblast cell measurements was 11.9%; the same percentage for the body weight gain was 26.3%. If the premise is made that 10% is a reasonable percentage error to expect in bio-assay work, it is found that to obtain this degree of error for both methods of assay the odontoblast method requires only 11 animals per level of ascorbic acid, whereas the growth method using male animals only requires 59 animals per level.

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All data reported in this thesis were obtained from animals following a 56 day experimental period. Key and Elphick (1931) used only a 14 day test period. They found sufficient changes had occurred in the incisor teeth after this short period of time for them to be able to divide scurvy into five stages, ranging from full scurvy to full protection from scurvy. They also found that with a 14 day test period "the growth of a guinea pig cannot be taken as any indication of the antiscorbutic value of its diet."

Key and Elphick based their determination of scurvy on microscopic changes occurring in the teeth in addition to those occurring in the odontoblast cells. As a result it is not possible to state whether sufficient differences in the height of the odontoblast cells of guinea pigs on different levels of ascorbic acid would exist if a 14 day test period were used.

Dalldorf and Zall (1930) report that the incisor teeth of healthy guinea pigs receiving a basal diet plus mixed green feed grow at a maximum rate of .850 millimeters per day, while those receiving a basal diet with no source of vitamin C grow only .306 millimeters per day. In view of this, to obtain

measurements of cells that have formed after the animal has been placed on test, it would be necessary to have an experimental period of at least 23 days for those on the higher levels. If desired, this time could be shortened considerably for those on the lower levels.

CONCLUSION

The height of the odontoblast cells in the incisor teeth of guinea pigs has been proven to be very sensitive to the amount of vitamin C consumed, and offers a reliable and highly accurate criterion for the bio-assay of vitamin C. Under the experimental conditions reported in this thesis, the average height of the odontoblast cells ranges from 21 microns in those animals in the 0.25 milligrams of ascorbic acid per day group to 51 microns in the 2.0 milligrams per day group.

Some of the advantages of the method are:

(1) a reduction in the variation of results of different technicians arising from the subjective error, (2) a greater specificity for vitamin C than the increase in weight criterion, and (3) in contrast to the growth response there is no difference in the response of the odontoblast cells of males or females.

SUMMARY

An improved method of the incisor tooth assay of vitamin C involving the micrometric measurement of the odontoblast cells of guinea pig incisor teeth is described. The accuracy of this method as compared to other incisor tooth assay procedures and to the growth method is discussed.

A report of the post mortem lesions found in all animals on test is included in the appendix.

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A P P E N D I X

POST MORTEM RESULTS

Post mortem examination of each animal revealed pathological conditions of varying intensity and location. Fatty infiltration, as well as necrosis of the liver and kidney, was frequent.

The occurrence of the liver and kidney conditions is shown in Table 1. The presence of scurvy as judged by hemorrhages around the leg joints, in the axillae, peritoneum, or in the muscles or subcutaneous tissue over areas exposed to injury is also included for comparison. Hemorrhage constitutes the only criterion used to determine macroscopic scorbutus, due to the fact that beading of the ribs of varying degrees, but never very pronounced, was found in 43% of the animals above the 1.0 milligram level. Many of these were present in the levels of ascorbic acid that according to the measurement of the odontoblast cells showed no microscopic scorbutic changes.

Appendix Table 1.
Pathological Conditions Encountered in
Experiments No. 139 and No. 139A.

Organs Affected	Daily Dose of Ascorbic Acid (Milligrams)					
	0.25	0.5	1.0	2.0	4.0	8.0
Liver	56%	69%	94%	91%	94%	91%
Kidney	31%	40%	47%	39%	22%	44%
Scurvy	93%	44%	0%	0%	0%	0%

Steato-necrosis, a white, focal, degenerative change in the abdominal fat, excluding omental fat, was observed in 40% of 70 animals in Experiment No. 139A.

As little is known about the exciting cause of steato-necrosis and as it has been impossible to produce experimentally by dietary means, the data obtained from observations made in this experiment, in which it occurred unexpectedly, are presented in Tables 2, 3 and 4.

Appendix Table 2.
Percentage Occurrence of Steato-Necrosis on Different
Levels of Daily Intake of Ascorbic Acid.

0.25 mgm.	0.5 mgm.	1.0 mgm.	2.0 mgm.	4.0 mgm.	8.0 mgm.
0%	21%	36%	43%	60%	29%

Appendix Table 3.
Percentage Occurrence of Steato-Necrosis in Different
Locations in the Abdomen Fat.

Sublumbar Fat	Testicular Fat	Perirenal Fat	Sublumbar and Perirenal	Sublumbar and Testicular	Sublumbar Perirenal Testicular
72%	40%	32%	25%	18%	14%

The following is the percentage occurrence of steato-necrosis in those animals with and without additional vitamin A (carotene):

Appendix Table 4.

Vitamin A - 29%	No Vitamin A - 50%
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The suspicion of a vitamin E deficiency in the diet was confirmed when it was found that almost all of those animals not receiving additional vitamin E orally developed muscle degeneration, whereas all of those that did receive the supplement showed no macroscopic symptoms of the degeneration. No case of heart or intestinal muscle degeneration was ever observed in these animals.

Some of the animals not receiving additional vitamin E did not develop muscle degeneration. The distribution of muscle degeneration among these animals is shown in Table 5, including the apparent effect on muscle degeneration of the feeding of additional vitamin A to one-half the number of animals on test.

Appendix Table 5.
Percentage Occurrence of Muscle Degeneration on the
Different Levels of Ascorbic Acid Intake,
With and Without Vitamin A.

	Daily Dose of Ascorbic Acid (Milligrams)					
	0.25	0.5	1.0	2.0	4.0	8.0
Vitamin A	38%	75%	100%	13%	75%	63%
No Vitamin A	38%	88%	100%	63%	88%	38%

DISCUSSION

From data presented in Tables 1 and 2, it may be considered that the basal diet is either deficient or unbalanced in some factor or factors, or contains some toxic substance that is causing the fatty infiltration and necrosis in the liver and kidneys, as well as the steato-necrosis.

Due to the fact that the dietary ingredients are all from natural sources, the presence of a toxic substance that would cause the liver and kidney damage is not very likely. Because other deficiencies were found in this diet, it may be reasonable to assume that the liver and kidney lesions were due to an as yet unknown dietary defect. This assumption is further strengthened in the light of the report of Gavin, Patterson and McHenry (1943) on the effect of choline, inositol, lipocaic and cholesterol on the liver and body fat content.

As may be seen in Chart I, the occurrence of steato-necrosis is more frequent with each increase in the amount of vitamin C fed up to the 4.0 milligram level, and then drops again. This may be due to one or both of two factors.

The increased intake of ascorbic acid may be responsible either through direct action or through causation of a greater demand for some substance that is not supplied in sufficient quantity. Vitamin C literature does not record the occurrence of this condition, so it is unlikely that it is a result of a primary effect of vitamin C. The greater food consumption,

deposition of fat and growth rate of those animals on the higher levels of vitamin C may in some way be related to the degenerative process. Its non-occurrence on the 0.25 milligram level is due to the fact that no abdominal fat was present in these animals in which to observe the condition.

Fat soluble and fat transport substances required by the body might well be considered when it is seen that there is a marked difference in its occurrence in the vitamin A supplemented group to that in the non-supplemented group.

