An Improvement in the Quantitative Assay of the Antiscurvy Vitamin (C)*

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WHILE the experimental data presented in this paper have to do with a review of present methods of evaluating antiscorbutics and the claims of Höjer's methods as contrasted with the Sherman method (now generally in use in the United States), the subject has a very direct bearing on health problems and practices, for the method of Höjer assumes a very specific response of teeth to presence or absence of vitamin C. That this response is specific and supports the suggestions for dental nutrition practice, first emphasized by Dr. Percy Howe and more recently by Hanke and Wells, will appear as we outline our experimental findings obtained in parallel studies of vitamin C assay methods.

The Sherman method[1] was first presented in 1922. LaMer, working with Sherman and Campbell, developed a diet for guinea pigs which was markedly superior to the hay-oats-water diet, up to that time generally used to produce experimental scurvy in these animals. Its superiority was based on evidence, considered satisfactory at the time, that it contained all the nutritional factors necessary for normal growth except vitamin C. Guinea pigs of 300 gm. on this diet developed acute scurvy and died of that disease in from 25 to 30 days. The smallest amount of addendum necessary completely to prevent scurvy for a period of 90 days when added to this diet in daily doses, was called the minimum protective dose, and since that dose was inevitably expressible only in amount of food carrying the same, Sherman's unit quantity of antiscorbutic, like that of Holst, was defined as the smallest quantity of food source necessary to total absence of scurvy symptoms in a 90-day test using guinea pigs and the above basal diet.

Since 1923, Kohman and I have used the Sherman method[2] in studying a wide range of fresh and canned vegetables and fruits.

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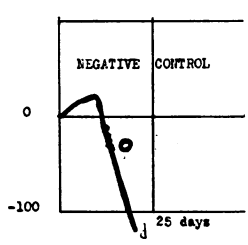
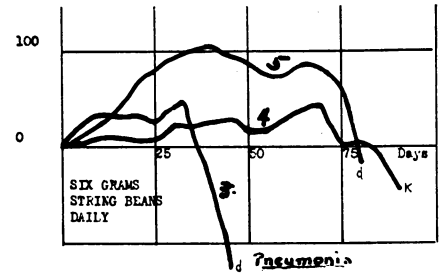


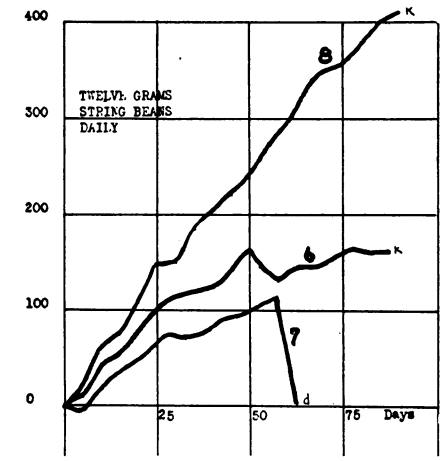
Brittleness	Pig 0.
Bones	***
Jaw	***
Teeth	***
Beading	***
Hemorrhages	
Muscle	***
Joints	***
Ribs	***
Intestines	***

BASAL DIET USED

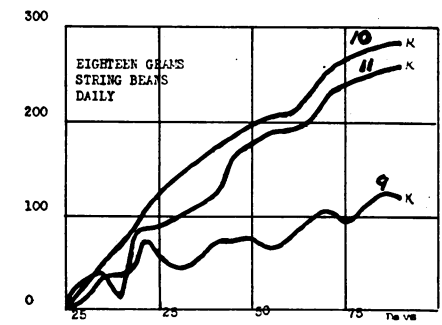
ROLLED OATS	50 PARTS
WHEAT BRAN	9 PARTS
BAKED SKIM MILK PWD.	30 PARTS
BUTTER FAT	8 PARTS
COD LIVER OIL	2 PARTS
SODIUM CHLORIDE	1 PART
TOTAL	100 PARTS



Brittleness Pigs	3	4	5
Bones	*	*	*
Jaw	*	*	*
Teeth	*	*	*
Beading	*	**	*
Hemorrhages			
Muscle	*	*	*
Joints	*	*	0
Ribs	*	*	*
Intestines	*	0	0



Brittleness Pigs	6	7	8
Bones	*	*	0
Jaw	0	0	0
Teeth	0	*	0
Beading	*	*	0
Hemorrhages			
Muscle	*	*	*
Joints	0	*	0
Ribs	0	*	0
Intestines	0	**	0



Brittleness Pigs	9	10	11
Bones	*	0	0
Jaw	0	0	0
Teeth	0	0	0
Beading	0	0	0
Hemorrhages			
Muscle	0	0	0
Joints	0	0	0
Ribs	0	0	0
Intestines	0	0	0

FIGURE I—A TYPICAL SHERMAN-LAMER TEST. Weight curves are shown of guinea pigs on various intakes of canned string beans and also the autopsy scores of each of the pigs on these diets. By this method we obtained complete protection from scurvy symptoms on a dosage of 18 gm. of canned string beans daily. In this series we used the basal diet given.

Statistically our numbers of test animal records now run into the thousands and provide material for some comparative observations of interest and profit. During the past year, therefore, my laboratory has devoted considerable time to such critical review of existing data and, through the coöperation of Dr. Gilbert Dalldorf of the Department of Pathology of the New York Hospital, we have extended our review by new experiments aimed to evaluate criticism of the Sherman method and of some of the substitutes offered, notably that of Höjer.[8]

IS THE ORIGINAL SHERMAN-LAMER BASAL DIET COMPLETE IN ALL GROWTH FACTORS EXCEPT VITAMIN C?

Their basal diet as given in 1922 consisted of the following:

	Per cent
Ground whole oats	59
Baked skim milk powder	30
Butterfat	10
Salt (NaCl)	1

We are using today the following modification of this diet:

	Per cent
Baked skim milk	30
Butterfat	9
Salt	1
Rolled oats and wheat bran (½ & ½)	59
Cod liver oil	1

plus a certain amount of yeast fed separately.

The bran was added to supply roughage in greater quantity, the cod liver oil as insurance against vitamin D deficiency, and in certain cases we had direct evidence of lack of one or more of the vitamin B factors, making yeast of value in supplementing the oats and milk content of these factors.

Sherman introduced a scurvy symptom score based on autopsy findings. Figure I shows the nature of this score and its use in determining minimal protective dosage of a tested antiscorbutic.

In spite of score and correction of basal diet defects the test has been criticised. The experimental pathologists took a hand in the matter. In his treatise on "Scurvy," Hess has adequately summarized the pathology of the disease up to the date of publication (1920). So far as I can find, Jackson and Moore,[4] 1916, were the first to note scorbutic changes in teeth. These observations were confirmed by Zilva[5] in 1919, but greatly extended by Höjer[8] in 1924. Aschoff and Koch[6] in 1919 also turned their attention to the pathology of scurvy,

and it is their viewpoint as to the function of vitamin C that led to the theory advanced by Wolbach and Howe[7] in 1926. We will return shortly to this work of Wolbach and Howe and their theory. From the viewpoint of methodology in assay, the work of Höjer especially concerns us. This Swedish pathologist supplemented his first studies, published in 1924, by a scheme for utilizing tooth pathology as a quantitative measure of antiscorbutic value. His scheme was published in detail in 1926.[8] It was critically reviewed by Goettsch and Key[9] in 1928. The following quotations from the 1926 paper of Höjer explain briefly why this scheme constitutes both a challenge of the Sherman method and a claim for efficiency requiring study:

> Sherman and others, in 1922, therefore proposed to limit the experimental time to 70 or 90 days and then to make a macroscopic post-mortem examination to make sure that there were no internal hemorrhages or other signs of scurvy. Sherman also introduced the rating of the scorbutic signs in animals which were not fully protected, and thus he estimated the dose of antiscorbutic given in quarters of the fully protective dose. Even with these improvements of Sherman's this method of determining the fully protective dose is very inaccurate, and often gives variations of 100 per cent or more.
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> . . .
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> Naturally a biological method cannot afford the accuracy or the simpleness of a chemical one. It is indeed the lack of a chemical method for the determination of the antiscorbutic value that has called in experimental pathology as a temporary aid to the physiologist. As a biological method, and in comparison with that hitherto used, my method has the following advantages: First, it is accurate in fixing the fully protective dose. This is of value particularly in physiological work. Secondly, the method requires only 3 weeks instead of 3 months. This is an essential factor in hygienic work. Lastly, being shorter, it is more economical than the older methods.

What is this method? In brief it rests on the claim that the teeth show specifically in 3 weeks or less the absence of an adequate amount of vitamin C in the diet. Höjer's own description[8] of his technic follows:

> As previously, young guinea pigs from a certain day on are given a basal diet free from antiscorbutic, but otherwise complete, and to this diet are added quantitatively daily doses of the juice to be examined. For a sharp determination of the fully protective dose it is advisable to have several animals on different doses. In addition there ought to be two control animals on the basal diet alone, and two on the basal diet with a fully protective dose of a known antiscorbutic. After a period of 10–14 days all the animals are killed. The one-half of the lower jaw is taken out and decalcified in a 5 per cent tri-chloracetic acid solution. It is then embedded and sectioned after 1 week. The section is made through the foremost molar at right angles to the longitudinal axis of the jaw. Some sections are stained with hematoxylin-eosin, and others with tri-oxyhematin according to the method of Hansen.

Such sections according to Höjer not only reveal histological pictures characteristic of scurvy or its absence, but it is possible from pictures of partially protected animals to predict the fully protective dose, as shown in Table I.

TABLE I

DETERMINATION OF THE ANTISCORBUTIC VALUE OF FOOD BY HÖJER'S METHOD[8]

	Part of Protective Dose
1. Dentine of normal size; its inner and outer layer uniformly colored; predentine regular, uncalcified; dentine and predentine holding collagen—	
a. Odontoblasts long, slender, parallel, of equal height	1.0
b. Odontoblasts partly shorter	0.9
2. Dentine very thin, uniformly colored; odontoblasts parallel, short (The degree of osteoporosis, hyperemia and collagen permits the distinguishing of different stages, but not very sharply. Those animals whose dentine formation seems to have been arrested are therefore excluded.)	0.8–0.3
3. Dentine in inner and outer layer differently colored.	
a. Odontoblasts on the larger pole of the incisor cross-section short and parallel	0.8–0.3
(1) The Tomes' canals going parallel through a normal predentine	0.8
(2) The uncalcified predentine defective formation of network bone beginning	0.7
(3) Uncalcified predentine lacking	0.7–0.5
(4) Network bone formation greater	0.4
(5) Tomes' canals in the old dentine widened	0.3
b. Odontoblasts on the larger pole of the incisor cross-section no longer in continuous layer	(0.3–) 0.2–0.0
(1) Tomes' canals in the outer layer of new bone	0.2
(2) No Tomes' canals in the new bone, osteoporosis and hyperemia well developed	0.1
(3) The old odontoblasts in greatest disorder, osteoporosis and hyperemia very evident	0.0

A tooth section showing the picture 3, a, (1), with 0.5 gm. of antiscorbutic daily would permit prediction that the fully protective dose of that antiscorbutic would be 0.622 gm. (0.5 gm. = 0.8x).

The value of Höjer's method obviously depends upon substantiation of claims for specificity. Do the tooth changes he reports result solely from the absence or inadequacy of vitamin C in the diet? Are they affected in any measure by absence or presence of vitamin D? Can we rely on the pictures of partial protection he presents to predict full protective doses?

To date two critical studies support his claim that tooth changes picture specific and early response to absence or inadequacy of vitamin C.

Goettsch and Key[9] have reported comparisons of Höjer's method with the older type. They studied particularly the question of whether the tooth changes noted had any correlation with symptoms of rickets and found none. The actual tooth changes were as de-

TABLE II

TABLE AFTER GOETTSCH AND KEY[9]

No. Guinea Pigs	Line Test	Blood serum mg. Ca per 100 c.c.	Mg. P per 100 c.c.	Per cent ash of dried and extr. femurs	Diagnosis
18	No rickets	9.9	5.8	59.4	Death by scurvy
24	"	11.3	5.3	55.0	"
28	"	12.0	5.3	57.9	"
35	"	9.4	5.0	56.2	Partly protected by 0.5 c.c. orange juice
36	"	10.1	5.0	57.4	Partly protected by 0.5 c.c. orange juice
38	"	10.9	5.0	59.0	Partly protected by 0.5 c.c. orange juice
31	"	9.8	5.0	54.2	Completely protected by 3 c.c. orange juice
32	"	9.1	5.0	56.2	Completely protected by 3 c.c. orange juice
34	"	9.0	4.5	56.2	Completely protected by 3 c.c. orange juice

scribed by Höjer and in their hands confirmed his criticism of the Sherman method, for whereas the Sherman method fixed 1.5 c.c. as the protective dose for orange juice, prevention of the scorbutic symptoms in teeth required a daily dosage of 3 c.c. of orange juice. Their only stricture is the reliability of prediction from the results with partially protected animals and they feel that individual variation in animals calls for fairly large numbers on these intermediate tests if the predictions as to full dosage are to be relied upon.

Wolbach and Howe[7] gave more attention to Höjer's histology and his theory of its cause than to his use of the study of antiscorbutic testing. They say:

> We do not question the accuracy of Höjer's description. His findings are different in important respects from ours, as we found in the state of complete scorbutus no formation of " osteodentin " or pulp bone. We believe that his diets were not completely deficient, because we obtained both conditions answering to his descriptions only in guinea pigs fed alternately on deficient and normal foods. . . . Our results corroborate completely and extend the deductions of Aschoff and Koch, so that we characterize the scorbutic state as one due to the inability of cells of supporting tissues to produce intercellular substances and to maintain existing intercellular substances.

Later in their paper[7] they further hold that Höjer's theory to account for changes as due to degeneration or the change of odontoblasts to osteoblasts is not proved. Rather they consider that the odontoblasts do continue to function, but through lack of a substance supplied by vitamin C their proliferated " jell " fails to set into dentine.

> . . . We therefore advance the theory that the failure of cells to produce intercellular substance in scorbutics is due to absence of an agent common to all supporting tissues which is responsible for the setting or jelling of a liquid product.

The immediate purpose of the present paper is to make clear the possibilities offered by the Höjer test for rapid assays of antiscorbutic values and to present evidence from our own studies of its validity. To that end we have devoted considerable time during the past year to checks and comparisons with our previous assays and also some study of material hitherto unpublished. We report here three such specific comparisons.

I. CANNED STRING BEANS TESTED BY BOTH METHODS

In Figure I is presented a series of studies of canned string beans by the Sherman method. Using both growth curves and scoring system this study showed 18 gm. of canned string beans per day to be the minimum protective dose. This series was then repeated using the Höjer procedure in part. Figure II represents tooth sections obtained in this study.

Photograph A of Figure II shows a magnified tooth section from an animal receiving 18 gm. string beans daily at the end of 30 days on the diet. (This was the protective dose by the Sherman method.) Even in this period the beginnings of scurvy are evident, though slight. The odontoblasts are irregular and beginning to separate from the dentine. A layer of jelly is beginning to form. Photograph B shows a much more distinct disturbance in tooth structure. The period on diet is the same (30 days), but the dosage of antiscorbutic was only one-third as much (6 gm. string beans daily). True dentine is markedly reduced. The odontoblasts are very irregular and have formed much false dentine. This material was partly bone-like, tending to support Höjer's claim that the character of the odontoblast output is actually changed in quality.

Photograph C is given to show the odontoblasts in higher magnification, and photograph D shows that the effect of inadequate antiscorbutic was already manifest in 20 days on the diet. With this method we were unable to reach a dosage of string beans that completely protected teeth. Even 18 gm. per day had already become difficult to use without reduction of the basal intake below efficiency in other factors.

The series shows clearly, however, that if the tooth is a true index of antiscorbutics, it is a much more sensitive one than the Sherman scoring system; that tooth protection demands more antiscorbutic than does the prevention of external symptoms of scurvy. This particular study definitely confirmed the claims of Höjer and others that the guinea pig's tooth responds very promptly to variation in vitamin C.

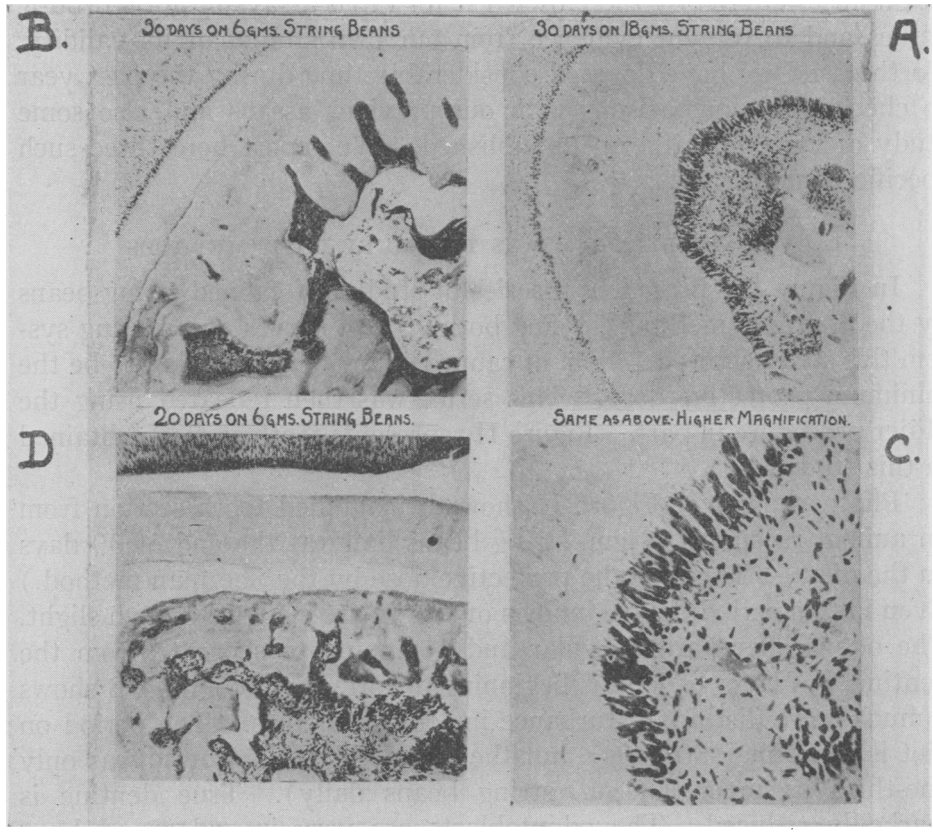

FIGURE II—TYPICAL HÖJER TEST RESULTS

In this series tooth sections were made at the end of 20 and 30 days on basal diet plus different intakes of canned string beans. Photographs A, B, C, and D are microphotographs of tooth sections from typical animals.

Photograph A—This shows the tooth of a guinea pig that had received for 30 days a daily dosage of 18 gm. canned string beans. The tooth shows slight scurvy. The broad band of dentine is separated from the cells which form it (odontoblasts) by a narrow band of jell-like material.

Protograph C—This shows a region of the section given in A highly magnified to show the soldier-like row of odontoblasts, the dentine with its fine striations of Tomes canals and the beginnings of the separation layer between odontoblasts and dentine.

Photograph B—In contrast to the above note in this section the very narrow band of dentine, the complete confusion of arrangement of the odontoblasts, the soldier-like rows are gone and the cells are in irregular islands in the pulp cavity. Between islands and dentine is a mass of semi-solid jell quite unlike true dentine. This effect was produced by inadequate antiscorbutic, 6 gm. canned string beans instead of 18 gm. daily.

Photograph D—Note that even in 20 days the effect of inadequate antiscorbutic is plainly evident.

II. ORANGE JUICE SERIES

During the past year we were asked to compare the antiscorbutic value of California and Florida oranges. Picking by size in the open market we carried out the regular Sherman test. Both types proved by this method fully protective in daily doses of 1.5 c.c., neither showing any appreciable superiority as an antiscorbutic over the other. This series, however, also permitted us at the same time to check Goettsch's[9] criticism of Höjer's method as well as to compare again Höjer's method with Sherman's in our laboratory. The check with Goettsch was remarkably good. In our hands 3 c.c. of either California or Florida orange juice was necessary to prevent tooth changes characteristic of scorbutus, exactly the relation obtained by Goettsch in her comparison (see Figure III, photographs A and B).

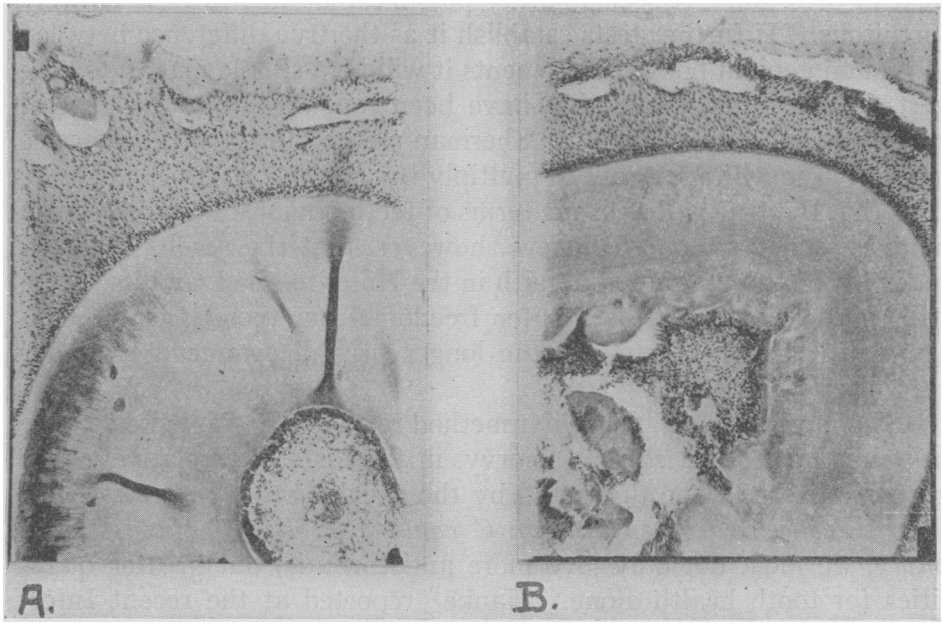

FIGURE III—THE EFFECT OF ORANGE JUICE ON TOOTH FORMATION

Photograph A—Tooth section from guinea pig on 3 c.c. of orange juice daily for 20 days. Note normal thick dentine, perfectly regular row of odontoblasts in direct contact with lower edge of dentine.

Photograph B—Tooth section from guinea pig on only 2.5 c.c. of orange juice daily. for 20 days. Note that 0.5 c.c. difference is plainly shown in disturbance of tooth structure. Odontoblasts have become irregular and begun to separate from dentine.

The tooth therefore is quickly responsive to very slight differences in antiscorbutic dosage.

III. BANANA ANTISCORBUTIC VALUE BY HÖJER METHOD

In 1926[10] I reported to this Association a study of the antiscorbutic value of the banana. Five gm. daily was the minimum protective dose determined by the Sherman method at that time. We have now retested the banana by the Höjer method. In doing it we introduced a modification suggested by Dr. Wolbach, that is, to include as positive controls in each series one animal fed for 5 days on a recognized adequacy of antiscorbutic following his 16 days on the banana intake. This procedure enables one to judge with more exactitude as to when the fully protective intake is reached. Figures IV and V show the tooth results.

Exactly as in the case of orange juice, the antiscorbutic dose of banana for tooth protection was found to be double that previously obtained as minimum protective dose by the Sherman method, 10 gm. instead of 5 gm. It seems hardly possible that this ratio is entirely fortuitous. If further tests establish it as the true difference between tooth and general body requirements it will be a simple matter to convert antiscorbutic values that have been reported since the general adoption in this country of the Sherman method into tooth protective values. We will need only to multiply by two.

What the true values are in terms of teeth obviously requires much more extended study. I believe, however, that the results reported herewith justify considerable faith in the Höjer method, and its speed, its relative simplicity, its greater freedom from the danger of contaminating disease factors in the longer test all commend it to our careful appraisal.

The facts on which the Höjer method rest have a wider significance in public health procedure. Scurvy, in fact, is still a relatively rare hospital disease when diagnosed by the usual signs. *If tooth health is so directly related to vitamin C content of diet as these studies imply,* we must not only give more antiscorbutic, but greater quantities for tooth health alone. Hanke[11] reported at the recent International Physiological Congress in Boston studies that confirm this view: He studied 100 cases, afflicted with every type of dental disorder, in subjects ranging from 4 to 60 years of age. He found the diets not markedly lacking in vitamin D, but *40 per cent were markedly deficient in vitamin C.* He says:

> It is possible, by means of a diet containing an abundance of vitamin C, to produce solid gum tissue, to arrest caries and, with the aid of prophylactic measures, to cure pyorrhea and induce bone regeneration in the alveolar tissue.

This view of Hanke's has been urged with much emphasis by Dr. Percy Howe.

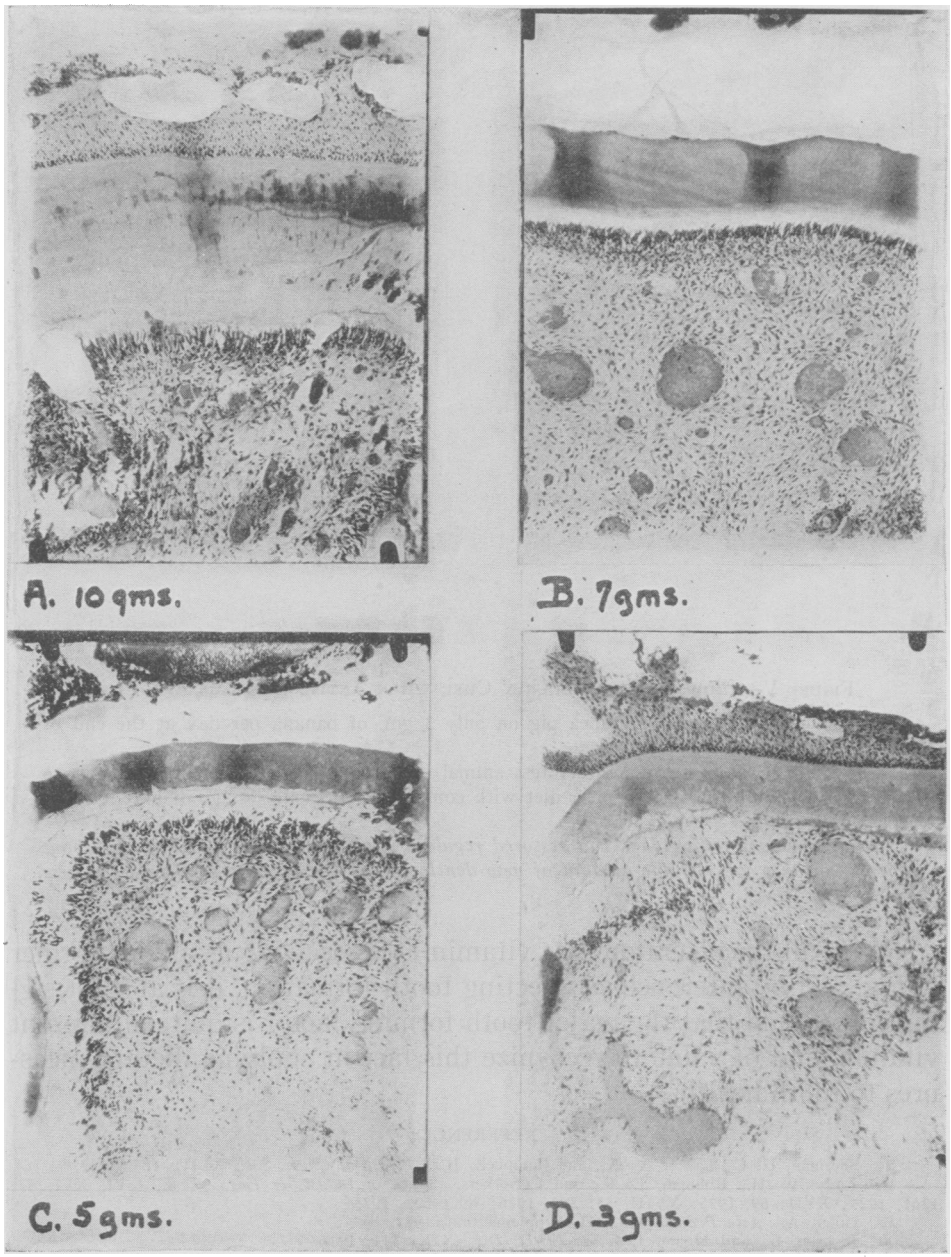

FIGURE IV—THE BANANA AS AN ANTISCORBUTIC

A—Ten gm. banana daily completely protective to teeth.

B—Seven gm. banana daily insufficient. Reduced dentine, odontoblasts still in regular rows but separated from dentine.

C—Five gm. banana daily gave still less dentine and beginning of irregularity in odontoblasts. This is the minimum protective dose by Sherman Method.

D—Three gm. banana daily shows complete derangement of odontoblasts.

As in the case of orange juice tooth protection requires double the amount of anti-scorbutic per day as was shown adequate against general scurvy symptoms by Sherman test and score.

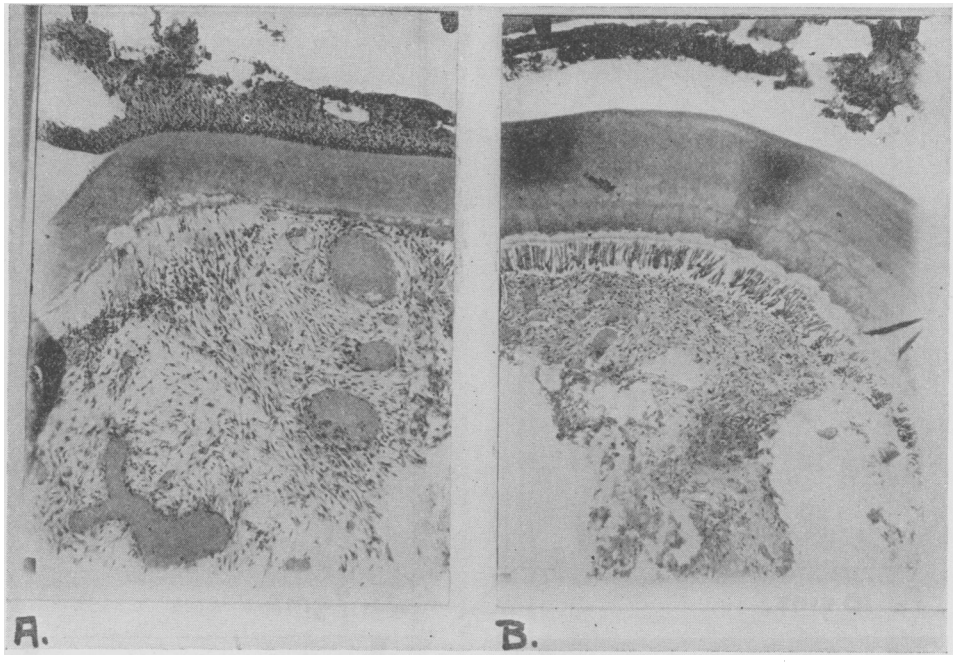

FIGURE V—SHOWS SPEED OF SCURVY CURE WHEN ANTISCORBUTIC IS ADEQUATE

A—Section of tooth of guinea pig on only 3 gm. of banana per day at the end of 21 days.

B—Section of tooth of another test animal who was fed 3 gm. banana daily up to the 18th day and then placed on a diet with completely adequate dosage of antiscorbutic for 5 days more.

Note contrasts. Five days had restored regularity to odontoblasts and brought about formation of a considerable amount of new dentine. See line of separation between new and old dentine.

With all the attention that vitamin D is receiving as a bone-former today, and by inference as affecting tooth formation, it is at least significant to find that the major tooth forming factor is quite a different vitamin, and of value to recognize this fact in applying dietary measures to tooth health.

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