STUDIES ON THE PRODUCTION OF RICKETS IN RATS AND THE MODE OF ACTION OF VITAMIN D

A Thesis

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By

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STATEMENT OF THE PROBLEM

Although the various phases of the work on vitamin D and rickets have attracted much attention of investigators all over the world in recent years yet few attempts have been made to study the mode of action of this vitamin, and to modify the few classical rachitogenic diets used for experimental purposes. As a result, a great deal has been discovered about the chemical nature, the distribution and the clinical and commercial applications of vitamin D, but the exact nature in which vitamin D operates within the body in the prevention and cure of rickets is still unknown. Likewise, the rachitogenic rations recommended by early workers may prove to be satisfactory in some cases, but wide variations and irregularities have also been reported by different laboratories where such rations are employed for producing experimental rickets. With these in mind, the following studies have been undertaken in the hope that some light might be thrown upon the problems just mentioned.

For convenience in presentation, the results of the work have been divided into two major parts, each of which has been again redivided into two subdivisions according to the nature of the work. Part I is concerned with the production of rickets in rats while Part II represents some studies made on the mode of action of vitamin D.

PART I

STUDIES ON THE PRODUCTION OF RICKETS IN RATS

A. SOME MODIFICATIONS IN THE STEENBOCK RACHITOGENIC RATION 2965

HISTORICAL AND INTRODUCTION

Since the publication by Harris and Bunker (1931, 1934, 1935) on the "variability in the corn component of a rachitogenic diet" many similar studies have appeared in the scientific literature during recent years. Holmes and Tripp (1933) analyzing samples of rachitogenic rations (Steenbock ration 2965) from different laboratories, found variations in ash, Ca and P contents and in the Ca:P ratios. Samples of ground maize from representative milling concerns showed considerable variation in fineness, ash, Ca and P contents, and in Ca:P ratios. Studying the effects of cereals and common salt on the development of rickets György (1933) concluded, that the kind of cereal and the amount of NaCl in the diet are important factors in determining the development of rickets in rats. Zucker, Hall, Mason and Young (1933) believed that in order to obtain good results in vitamin D assay, rats used for the tests must have good growth during the testing period so that complications and the loss of animals may be avoided. These authors devised a rachitogenic ration which will give growth comparable with that of a good stock diet, during the three weeks of experimentation. In a series of publications Steenbock and co-workers (Templin and Steenbock, 1933, 1 and 2; Lowe and Steenbock, 1936, 1 and 2) reported that germinated autolyzed maize, immature maize or maize treated with HCl were less rachitogenic than mature maize

owing to an increased content of inorganic P. In another paper Thomas and Steenbock (1936) stated that no significant difference was observed in the growth produced or in the severity of rickets produced by any of the cereals (maize, whole wheat, patent flour and polished rice). Nijveld (1936) found that the addition of dried liver or of liver extract to the Steenbock ration 2965 caused improved appetite and growth in young rats. These beneficial effects did not interfere with the development of rickets, and prevented loss of test animals by intercurrent diseases during experiments.

From these studies two generalizations may be drawn concerning the qualities of a satisfactory rachitogenic ration. In order to produce experimental rickets in rats successfully, the rachitogenic diet used must posses the following two qualities:

1. The corn (or other cereal) component must have a constant chemical composition which is favorably rachitogenic.
2. Enough growth-promoting factor must be present in the rachitogenic diet so that the rats may have a proper gain in weight during the experimental peridd.

In view of the fact that there is a possibility that ground whole corn may contain variable amounts of vitamin D which would affect the degree of rickets produced on a diet containing such a corn it was considered desirable to attempt to develop a

rachitogenic diet using granulated yellow corn or table corn meal. Since in the preparation of the latter most of the bran is removed, any vitamin D which might have been formed in the outer layers of the corn kernel due to the exposure of corn to sunlight during harvesting would be eliminated. Using the Steenbock ration 2965 formula as a basis granulated corn was substituted for whole corn meal and certain other modifications were made which were intended to yield a satisfactory rachitogenic diet.

EXPERIMENTAL

The original Steenbock ration 2965 as well as six modified rations were used in this experiment. The rations and their modifications are as follows:

Ration No.	Modifications
I.	Steenbock ration 2965, no modification
II.	Same as I using granulated corn
III.	Same as II plus 1% dried yeast
IV.	Same as II plus 5% linseed meal
V.	Same as II including 25% ground oats
VI.	Same as V plus 1% dried yeast
VII.	Same as V plus 5% linseed meal

Note: Wherever yeast, linseed meal, or ground oats were introduced into the ration, corresponding amounts of corn were reduced.

Ten young rats approximately three weeks of age weighing 50 to 60 grams were placed on each ration. The usual care in obtaining uniform distribution of rats with regard to litter and sex was observed. At the end of three weeks, animals 1-3 of each group were given 2.7 U. S. P. units of vitamin D standard reference oil while animals 4-7 received a supplement of M. S. C. vitamin D milk to supply the same amount of vitamin D. Animals 8-10 received basal rations only. All animals were killed at the end of ten days after the beginning of the supplements. The radii and ulnae were removed for line test and femura, for total ash analysis.

After having been preserved in 95% alcohol for at least 24 hours the wrist bones were split and stained for 2 minutes in a 2% $AgNO_3$ solution. The stained bones were then washed and immersed in distilled water and were exposed to either natural or artificial light until a desired darkness in the calcified area of the bone had been developed. As soon as this was achieved the bones were fixed in a 10% sodium thiosulphate solution to prevent further change in color. A representative bone from each rat was selected for making the photographic plate.

The femura were extracted for 40 hours with 95% alcohol and the clean, dried bones were ashed in a muffle furnance, and their total ash content calculated.

Growth during the experimental period, first three weeks, ash content of femura, the general appearance of the line and the width of the metaphysis were employed as criteria for the quality of a satisfactory rachitogenic ration.

RESULTS AND DISCUSSION

Results of the growth response of the animals during the three-week experimental period are presented in Table I and Graph I while those of the line tests and bone ash determinations are presented in Plate I and Table II respectively.

When granulated corn was substituted for ordinary corn in the Steenbock ration 2965 the growth rate of the animals was greatly reduced. Rats fed Ration II made an average gain of only nine grams in three weeks. A growth-promoting factor was evidently lacking in the granulated corn. This was believed to be due to the removal, during the process of milling of the granulated corn, of both the germ and the outer hull of the corn grain. A deficiency in vitamins B and G and of essential amino acids in the granulated corn seemed to be the main cause for poor growth. When 1% yeast (Ration III) or 5% linseed meal (Ration IV) or 25% oatmeal (Ration V) was added, growth of the test animals was improved slightly in each case. The animals made average gains of 16 grams, 18 grams and 19 grams respectively, during the three week period. Since best results in vitamin D assay are obtained from animals which will gain 25-35 grams at the end of three weeks after they have been placed on a

rachitogenic ration the above improvements were not considered sufficient. The most satisfactory rachitogenic ration in this experiment, from the standpoint of promoting growth was found to be Ration VI in which both dried yeast and oatmeal were added. Animals fed Ration VI gained an average of 33 grams in three weeks. Ration VII was the second best as far as the promotion of growth is concerned. Animals fed this ration gained 28 grams.

Plate I shows the results of the line tests. If Steenbock ration 2965 (Ration I) is considered as a standard it may be seen that at least two other modified rations gave equally good or better results. When the width of the metaphysis, the size of the bone, the uniformity, location and general appearance of the line are all considered Ration VI proved to be definitely superior while Ration VII was equally as good as the Steenbock Ration (Ration I). Other rations were considered unsatisfactory because of the inferior lines produced in response to feeding vitamin D.

Little difference was observed in the ash content of femura of the test animals. Regardless of the ration fed the femura of the negative control or basal animals (animals 8-10) all had a very low ash content, indicating severe rickets. On the other hand, the total bone ash of all animals which had been supplemented either with vitamin D standard reference oil or with vitamin D milk was increased slightly in each case. No comparisons could be made from the bone ash determinations alone in this experiment, as to which of the rachitogenic rations was superior for use in vitamin D assay. Other factors must also be considered.

Table I

Growth Response of Young Rats Fed Various Rachitogenic Rations

(All weights are averaged from 10 animals)

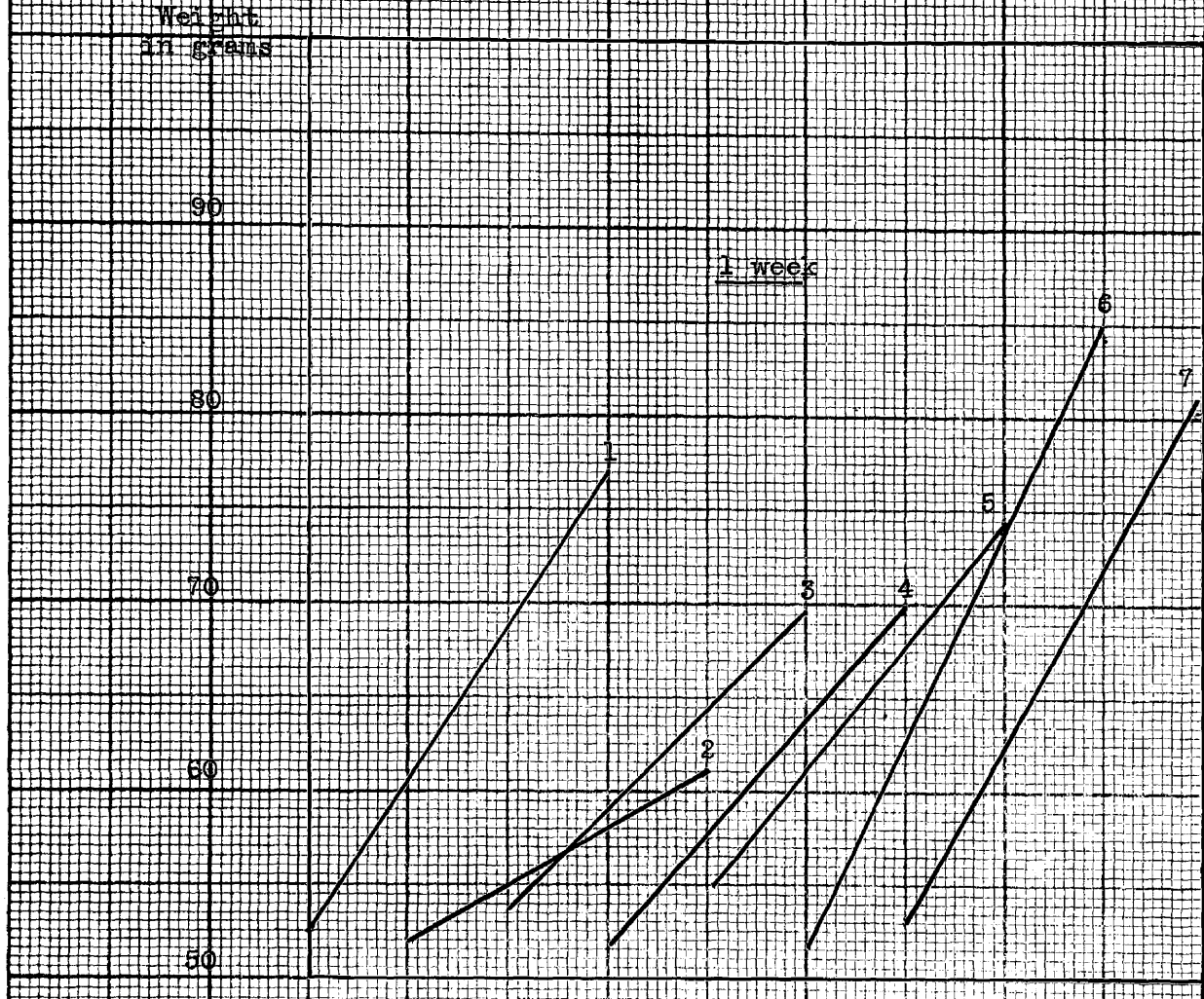
Ration	Initial Weight	Weekly weights			Wt. gained in 3 weeks
		1st week	2nd week	3rd week	
	Grams	Grams	Grams	Grams	Grams
I. Steenbock Ration 2965	53	60	68	77	24
II. Same as I using granulated corn	52	53	58	61	9
III. Same as II plus 1% yeast	54	55	63	70	16
IV. Same as II plus 5% linseed meal	52	55	63	70	18
V. Same as II plus 25% oatmeal	55	58	66	74	19
VI. Same as V plus 1% yeast	52	60	73	85	33
VII. Same as V plus 5% linseed meal	53	60	71	81	28

Graph I

Growth Reponse of Young Rats Fed Various

Rachitogenic Rations

(An average of 10 animals each)



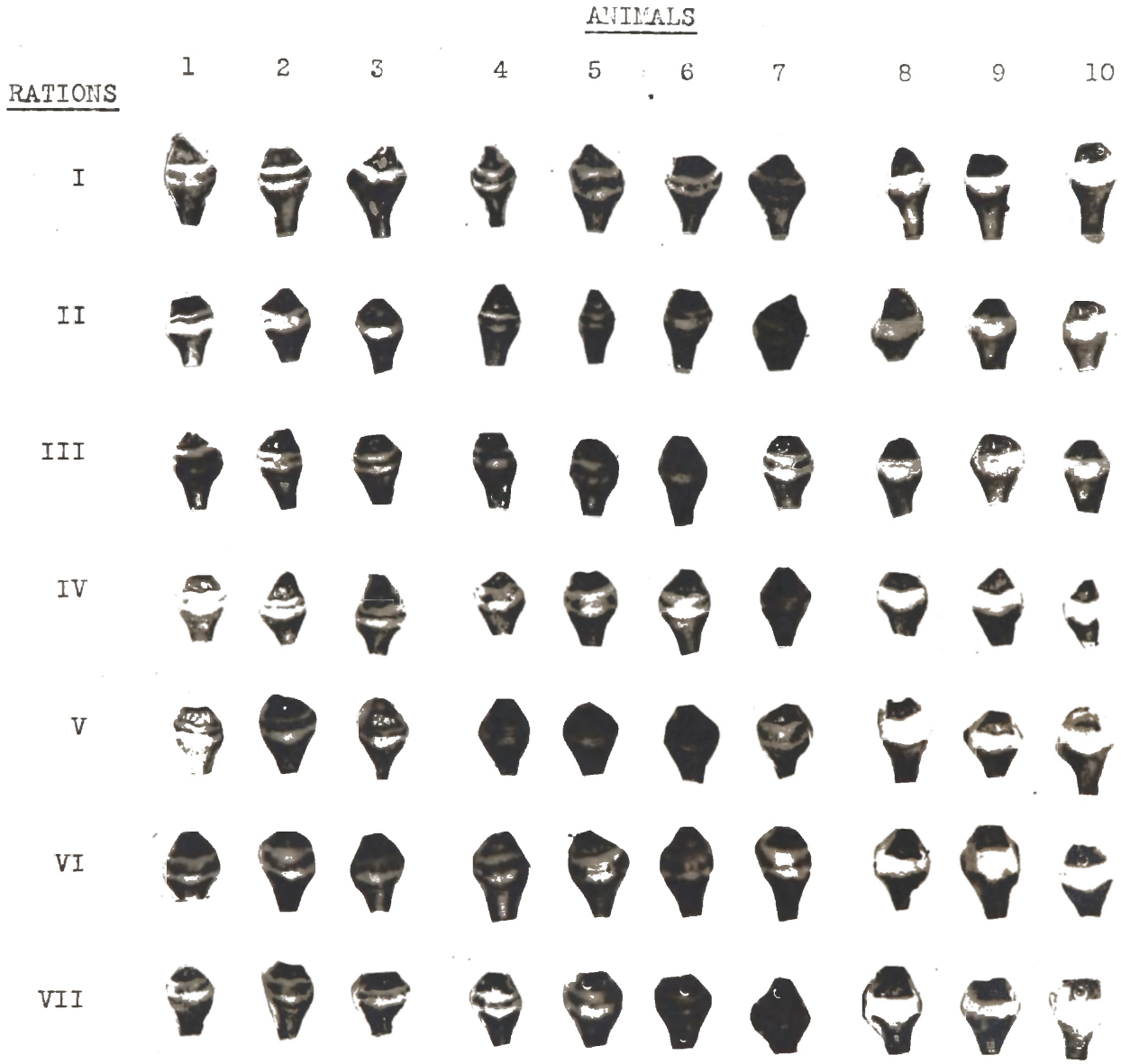
1. Steenbock ration 2965
2. Same as 1 using granulated corn
3. Same as 2 plus 1% yeast
4. Same 2 plus 5% linseed meal
5. Same as 2 plus 25% oatmeal
6. Same as 5 plus 1% yeast
7. Same as 5 plus 5% linseed meal

Table II

Percentage of Bone Ash of Rats Fed Various Rachitogenic Rations

Rations	Supplements		
	Vit. D Standard Reference Oil (3 rats)	Vitamin D Milk (4 rats)	Basal Ration Only (3 rats)
I. Steenbock Ration 2965	29.5	24.1	22.2
II. Same as I using granulated	24.1	23.8	20.0
III. Same as II plus 1% yeast	26.5	27.2	23.2
IV. Same as II plus 5% linseed meal	23.4	24.9	21.7
V. Same as II plus 25% oatmeal	25.2	22.9	20.5
VI. Same as V plus 1% yeast	25.9	25.1	21.0
VII. Same as V plus 5% linseed meal	23.0	26.4	21.6

Plate I. Results of Line Tests



Rations: I, Steenbock ration #2965; II, Same as I using granulated corn; III, Same as II plus 1% yeast; IV, Same as II plus 5% linseed meal; V, Same as II plus 25% oatmeal; VI, Same as V plus 1% yeast; VII, Same as V plus 5% linseed meal.

Animals: 1-3, supplemented with 2.7 U. S. P. units of vitamin D reference oil each; 4-7, received 18.9 cc. of M.S.C. vitamin D milk each which contains 2.7 U. S. P. units of vitamin D and 8-10, received basal rations only.

B. VITAMIN D CONTENT OF SUN-CURED AND IRRADIATED CORN, A PRELIMINARY REPORT

HISTORICAL AND INTRODUCTION

Since the irregularities reported by Harris and Bunker (1931, 1934) in the production of experimental rickets, many investigations have been conducted in various laboratories to find the variable factor in the rachitogenic ration. A brief review of literature pertaining to this subject was included in Part I Section A of this thesis. In general, most of the investigators on this problem were interested in the total P content, the partition of P compounds or the Ca:P ratios of the rachitogenic ration but none suspected the possibility of the variation of vitamin D content in its yellow maize component. Since corn is relatively rich in sterol content it can easily acquire antirachitic properties if sufficient activation is given, either through exposure to sunshine or through artificial irradiation. It is a general practice in many localities that during harvesting, the corn is husked in the field and left there for a period of several days before it is taken indoors for storage. Under such conditions there is reason to believe that such corn may receive enough ultraviolet irradiation from the sun to contain an appreciable quantity of the antirachitic vitamin.

Coward (1933) irradiated two samples of dried yeast by exposure to Indian sunshine for one hour (11:00 A.M. till noon on October 6, 1932) and found that one of the irradiated yeast samples

contained 5-20 I. U. of vitamin D per gram when tested biologically. Bechtel (1935) observed that parts of the corn plant (tassels, silk and the dry leaves on the lower part of the plant) that were sun-dried in the field at the time of ensiling were good sources of vitamin D, while dried material prepared from the upper green leaves of the corn plant was found to be devoid of the antirachitic factor. He also demonstrated, by means of rat assay, that the sun-dried material contained 1226-2449 U. S. P. units of vitamin D per pound, whereas the dried material prepared from the upper green leaves contained no vitamin D when so tested.

The purpose of this preliminary investigation was to secure data on the vitamin D content of sun-cured corn under conditions which are comparable to those in general farming practice.

EXPERIMENTAL AND RESULTS

Six bushels of yellow corn were collected on October 12, 1936 from the Plant Breeding Farm of the Michigan Agricultural Experiment Station. It was husked indoors, and divided into three equal batches of two bushels each. One batch was exposed to sunlight on clear fair days between the hours of 11:00 A. M. and 3:00 P. M. for a total of ten hours (October 15-27, 1936) while a second batch was irradiated under a mercury vapor lamp for one minute. A third batch kept and dried indoors was used as control.

Corn meals made by using these various corns were employed in preparing basal rations according to the formula of M. S. C. rachitogenic ration #2 which consists of: corn, 50; oats, 25; wheat gluten, 20; $CaCO_3$, 3; NaCl, 1 and yeast, 1.

Six standard young rats suitable for vitamin D assay were placed on each of the three basal rations. At the end of 30 days the animals were sacrified. Results of the condition of metaphysis, blood P, bone ash analysis, as well as, growth in each group are presented in Table III.

No appreciable difference was obtained when the basal rations were made up according to the formula of the Steenbock ration 2965 hence the results will not be included in this report.

Table III

Vitamin D Content of Sun-cured and Irradiated Corns

Basal Ration, M.S.C. Rachitogenic #2	No. of Animals	Initial Weight	Final Weight	Weight Gained	Condition of Metaphysis	Blood P	%Bone Ash
		Grams	Grams	Grams			
Sun-cured Corn	6	52	91	39	Rachitic	3.10	26.6
Irradiated Corn	6	53	101	48	Normal	3.85	42.9
Control Corn	6	53	89	36	Rachitic	2.01	22.4

DISCUSSION

Although no striking evidence was obtained in this preliminary investigation, the results offer some support of the view that under proper conditions of activation determined by season, locality, the time of exposure etc. it is possible to increase vitamin D content of corn by the process of sun-curing. The fact that rats fed the sun-cured corn had slightly higher blood P and bone ash than those fed the control corn indicates clearly that the sun-cured corn was definitely less rachitogenic than that of the control corn. Rats fed the irradiated corn did not develop rickets at all, as shown by the normal condition of the metaphysis, 3.85 blood P and 42.9% bone ash. The results of the irradiated corn were considered significant because they indicate the abundance of pro-vitamin D in the corn grain which can be easily activated. Since all the corn used for either sun-curing or ultraviolet light irradiation was left on the cob and no efforts were made to turn the cobs over during either process, the actual area of corn surface exposed was rather small. Considering the limited area of surface exposed in each corn grain, and the low intensity of ultraviolet rays in Michigan sunshine in October, it is surprising that even under such conditions some differences were observed between the sun-cured corn and the control corn in their rachitogenic properties. It is believed that any exposure to the ultraviolet irradiation from a strong sunlight, for an appreciable length of time, during the harvesting of corn may alter its vitamin D content considerably. If this is true the

irregularities reported by various investigators in the production of experimental rickets may be partially explained. Further work on this problem is being planned in order to obtain more conclusive results.

PART II

STUDIES ON THE MODE OF ACTION OF VITAMIN D

A. INTRODUCTION

Only a few references are available in the scientific literature on the mode of action of the antirachitic vitamin since its recognition by McCollum (1922). It has been proven beyond doubt that vitamin D is essential for normal Ca-P metabolism, for the development of sound bone and teeth and for the prevention and cure of rickets both in man and in animals, but the true mechanism in which vitamin D acts in regulating these important biological processes has never been made clear.

In 1926 Lewis and Zotterman first suggested that ultra-violet light forms in the skin a histamine-like substance which, presumably, would increase the gastric secretions. The healing action of vitamin D might be due to this increased gastric secretion. This theory was disproved recently by Daly (1933) who injected some histamine into rachitic rats and produced a great increase of secretion of gastric juice but no evidence of healing of the rachitic bones. Bauer and Maddock (1931) were also unable to explain the healing action of vitamin D on the basis of an increased formation of acid in the gastro-intestinal tract. Taylor, Brannon and Kay (1930) proposed that vitamin D functions by stimulating the parathyroid, but Harris, (1932) after investigating clinical hypervitaminosis, substantiated a theory that the characteristic mode of action of vitamin D is to permit an increased "net absorption" of Ca and / or P from the gut; tending to raise the level of the Ca and / or P, the latter rise, automatically brings about increased calcification in

sites provided with the "bone" or calcifying enzyme, phosphatase. Upon injection of a single large dose of viosterol (200,000 I. U. of vitamin D) to rabbits, Heymann (1936) found, that the rabbit serum was rendered antirachitically active. Daily intramuscular injections of 0.6 ml. of such serum cured rachitic rats in 8 to 10 days. The antirachitic substance (vitamin D) remained in the rabbit blood for two to three months. From the above evidence it is safe to assume that vitamin D operates in the blood stream regulating the "net absorption" or "net retention" of Ca and / or P rather than at the site of calcification. The present investigation was planned to obtain further evidence in support of this view.

B. THE EFFECT OF VITAMIN D ON FECAL ALKALINITY, FECAL N, BLOOD P AND pH OF THE ALIMENTARY CANAL IN BOTH YOUNG AND ADULT RATS

HISTORICAL

As early as 1924 Zucker and Matzner observed that the feces of rats, fed a rachitogenic diet, gave a pH of 7.4 to 8.0 when suspended in water. When such rats were given a supplement of cod liver oil, the reaction of the feces was lowered to about 6.2 while the feces of control rats, fed cotton seed oil, gave an unchanged alkaline reaction. Jephcott and Bacharach confirmed Zucker and Matzner's observation in 1926 and a few years later the same authors (Bacharach and Jephcott, 1929) recommended a method for vitamin D assay based on an examination of the fecal pH of rats on a rachitogenic ration. They found that the method was useful and satisfactory provided certain experimental conditions were observed. Redman (1929) found only a certain degree of correlation between pH and % of Ca in the feces of rachitic children. In studying the Ca-P excretion in rabbits receiving diets containing varying Ca:P ratios with and without irradiated ergosterol McGowan (1933) noticed a very striking effect of irradiated ergosterol in causing a shift of the P excretion from the feces to the urine in every case. This evidently may have an indirect effect on the total amount of Ca excreted through the feces. Nitzescu and co-workers (1933) found that treatment with ultraviolet light reduced the fecal Ca and P in children. Recently,

Friedman (1936) made a comparative study of the pH and bacterial flora of the feces of normal and rachitic rats and of rachitic rats healed with irradiated ergosterol or with vitamin D milk. He reported that during the development of rickets the pH of the feces of rats on Steenbock ration 2965 rose from 6.6 to 7.6. During healing the pH fell again to a normal level, 6.2-6.6. As far as is known, no studies have been made trying to correlate the total fecal alkalinity with the action of vitamin D. The purpose of the present experiment was to establish such a relationship.

EXPERIMENTAL

Eight different rations and both young and adult rats were employed for this experiment. The rations and their components are as follows:

1. Low Ca Ration - yellow corn, 39; ground oats, 39; wheat gluten, 20; NaCl, 1; plain yeast, 1 and $CaCO_3$, none.
2. With Vitamin D - same as 1 using irradiated yeast instead of plain yeast
3. 1% $CaCO_3$ Ration - Same as 1 including 1% $CaCO_3$
4. With vitamin D - Same as 3 using irradiated yeast
5. 2% $CaCO_3$ Ration - Same as 1 including 2% $CaCO_3$
6. With Vitamin D - Same as 5 using irradiated yeast
7. 3% $CaCO_3$ Ration - Same as 1 including 3% $CaCO_3$
8. With Vitamin D - Same as 7 using irradiated yeast

Owing to the tremendous amount of time consumed in this experiment, only two young white rats, 21 to 25 days of age weighing between 50 and 60 grams and one adult female rat weighing 200-350 grams were placed on each of the eight rations. Weekly food consumption and fecal excretion of each animal were recorded and the fecal samples were dried and saved for analysis. Care was taken to remove, as much as possible, the hair and food contaminations from the fecal samples before they were used for any of the determinations.

Determination of Fecal Alkalinity - For total alkalinity determination, a simple titration method was employed. One gram of dried, ground feces was placed in a 500 ml. Erlymer flask to which 50 ml. of a 0.1N H_2SO_4 or HCl had been added. The flask was allowed to stand for two hours at room temperature with occasional shaking. Then the content was filtered through an ordinary filter paper and 25 ml. aliquots were titrated against 0.1N NaOH using phenolphthalein as indicator. The total alkalinity of the feces was expressed as the No. of ml. of 0.1N alkali per gram of dried feces.

Fecal N and Blood P Determinations - Fecal N was determined by the Kjeldahl method while the blood inorganic P, by the Youngburg method described in Hawk and Bergeim's "Practical Physiological Chemistry", tenth edition.

Determination of pH of Alimentary Canal - The whole of the gastro-intestinal tract was removed, and placed in physiological salt solution immediately after the animal was killed by etherization.

The gastro-intestinal tract was then divided into seven portions as follows: The stomach, into the cardiac sac and the pylorus; the small intestine, into three equal portions; the cecum and the colon were taken as one portion each. The content of each of the seven divisions was emptied into a 50 ml. beaker containing 5 ml. of distilled water and the mixture was thoroughly stirred. Two or three ml. of the supernatant fluid was taken for pH determination using the quinhydrone electrode method. All pH determinations were completed within thirty minutes.

RESULTS AND DISCUSSION

Results on the growth, food consumption, fecal excretion etc. and on the fecal N, fecal alkalinity, blood P and on the pH of alimentary canal of young rats are presented in Tables IV-VII and Graphs II-III inclusive. Composite fecal samples from two rats within each group were taken for the fecal N and fecal alkalinity determinations while the determinations for blood P and pH of alimentary canal were made on individual rats.

Rats on the low Ca diet (Ration 1) grew poorly during the experimental period and seemed to have less ability to utilize their food as indicated by the high feces/food ratios. When vitamin D was added in the form of irradiated yeast (Ration 2), both growth and food utilization were considerably improved. In rations 3-6

the same general improvement was also observed. When the $CaCO_3$ level in the diet was raised to 3% of the total weight (Ration 7) vitamin D seemed to have little or no effect in improving the ability on food utilization of the test animals. This appeared to be due to the artefact resulting from the unusual food mixture in which 3% $CaCO_3$ was incorporated.

The blood P picture in the various groups was as expected and no significant changes were observed in any of the N determinations. pH values of the alimentary canal were in agreement with those found by previous investigators. These tests were included merely to check the experimental error and to eliminate other possibilities which might lead to wrong conclusions.

The total alkalinity of the feces of the test animals seemed to be directly proportional to the amount of $CaCO_3$ included in the ration. The feces from rats fed the low Ca ration had an average alkalinity value of 5.5; on the 1% $CaCO_3$ ration, 7.8; 2% $CaCO_3$ ration, 13.8 and 3% $CaCO_3$ ration, 24.0. When vitamin D was added to these same rations the alkalinity was reduced in each case. The average values were: 4.5, 6.7, 11.4 and 21.3 respectively.

Only the total fecal alkalinity determinations were made on the adult rats. These animals received the identical care as did the young rats. Their food consumption and feces excretion was recorded weekly and the fecal samples were prepared in the same manner for the alkalinity tests. Weekly fecal alkalinity values

of the various groups are presented in Table VIII while the average values for the entire experimental period are given in Graph IV.

The results of the total fecal alkalinity tests were considered significant because they seem to confirm the "net absorption" or "net retention" theory substantiated by Harris (1932). In both low Ca and high Ca rickets the ability of the animals to absorb Ca and / or P from the gut or to retain the Ca and / or P at a normal level in the blood stream appeared to be at a minimum. One of the functions of vitamin D is to remedy the above mentioned abnormal conditions in the animal organism. The characteristic action of vitamin D then, as originally stated by Harris (1932), and further confirmed by the present experiment, is to improve the "net absorption" or "net retention" of Ca and / or P resulting in an increased calcification of the bones.

Table IV

Growth, Food Consumption and Fecal Excretion of Young Rats Fed Various Rations with and without Vitamin D during a 4-week period

Ration	Animal	Weight gained	Food consumed	Feces excreted	Feces / Food* ratio
		Grams	Grams	Grams	
1 Low Ca ration	a	23	156	18	.115
	b	26	153	16	.105
2 Same plus irrad. Y.	a	83	270	26	.096
	b	71	282	27	.095
3 1% $CaCO_3$ ration	a	62	237	23	.099
	b	60	259	24	.093
4 Same plus irrad. Y.	a	85	278	24	.086
	b	79	287	27	.095
5 2% $CaCO_3$ ration	a	82	293	30	.104
	b	64	264	26	.100
6 Same plus irrad. Y.	a	89	284	30	.105
	b	66	260	24	.094
7 3% $CaCO_3$ ration	a	75	290	32	.112
	b	53	260	28	.109
8 Same plus irrad. Y.	a	96	314	34	.110
	b	89	302	33	.110

* Feces / Food Ratio = Total amount of feces excreted in 4 weeks / Total amount of food consumed in 4 weeks.

Table V

Blood Inorganic P and Total Fecal N of Young Rats Fed Various Rations with and without Vitamin D

Ration	Animal	Blood* P	% Fecal N **			
			1st week	2nd week	3rd week	4th week
1 Low Ca ration	a	4.7				
	b	4.5	4.0	3.4	3.6	3.6
2 Same plus irrad. Y.	a	5.1				
	b	5.2	4.6	4.3	4.1	4.1
3 1% $CaCO_3$ ration	a	4.3				
	b	4.0	4.5	3.6	3.5	3.8
4 Same plus irrad. Y.	a	4.8				
	b	5.5	4.6	3.6	3.6	3.8
5 2% $CaCO_3$ ration	a	4.5				
	b	5.3	4.8	3.8	3.6	3.8
6 Same plus irrad. Y.	a	6.3				
	b	5.9	4.5	4.0	3.8	3.6
7 3% $CaCO_3$ ration	a	5.3				
	b	3.8	4.0	3.5	3.2	3.5
8 Same plus irrad. Y.	a	5.3				
	b	5.1	4.3	4.0	3.8	3.6

* Milligrams of inorganic P per 100 ml. of blood.

** Values obtained by analyzing composite fecal samples of both rats within each group

Table VI

pH of Alimentary Canal of Young Rats Fed Various Rations with and without Vitamin D

Ration	Animal	Cardiac sac	Pylorus	Small intestine			Cecum	Colon
		1	2	3	4	5	6	7
1 Low Ca ration	a	2.56	6.40	6.60	6.84	7.70	6.68	6.43
	b	2.40	6.52	6.69	6.86	7.87	6.60	6.52
2 Same plus irrad. Y.	a	2.55	6.26	6.35	6.43	7.45	6.35	6.43
	b	2.29	5.84	6.60	6.94	7.20	6.60	6.10
3 1% $CaCO_3$ ration	a	2.71	6.52	6.76	7.03	7.78	7.10	7.37
	b	2.63	6.52	6.43	6.43	7.53	7.10	- -
4 Same plus irrad. Y.	a	2.29	6.52	6.43	7.03	7.37	7.11	7.37
	b	2.38	6.78	6.78	6.94	7.37	7.53	7.20
5 2% $CaCO_3$ ration	a	2.29	6.35	7.11	6.94	7.95	8.5+ *	7.94
	b	3.05	7.53	7.03	6.86	7.53	8.5+	8.04
6 Same plus irrad. Y.	a	3.30	6.86	6.69	6.86	7.57	8.5+	7.70
	b	3.30	6.69	6.52	7.03	7.61	7.53	7.94
7 3% $CaCO_3$ ration	a	2.89	7.11	7.70	7.53	7.87	8.5+	7.78
	b	3.66	6.86	7.62	8.21	8.46	8.5+	8.04
8 Same plus irrad. Y.	a	3.83	7.30	7.45	7.37	8.29	8.5+	7.53
	b	3.47	7.03	7.70	7.62	8.50	8.38	7.45

* All pH values higher than 8.50 are indicated as 8.5+.

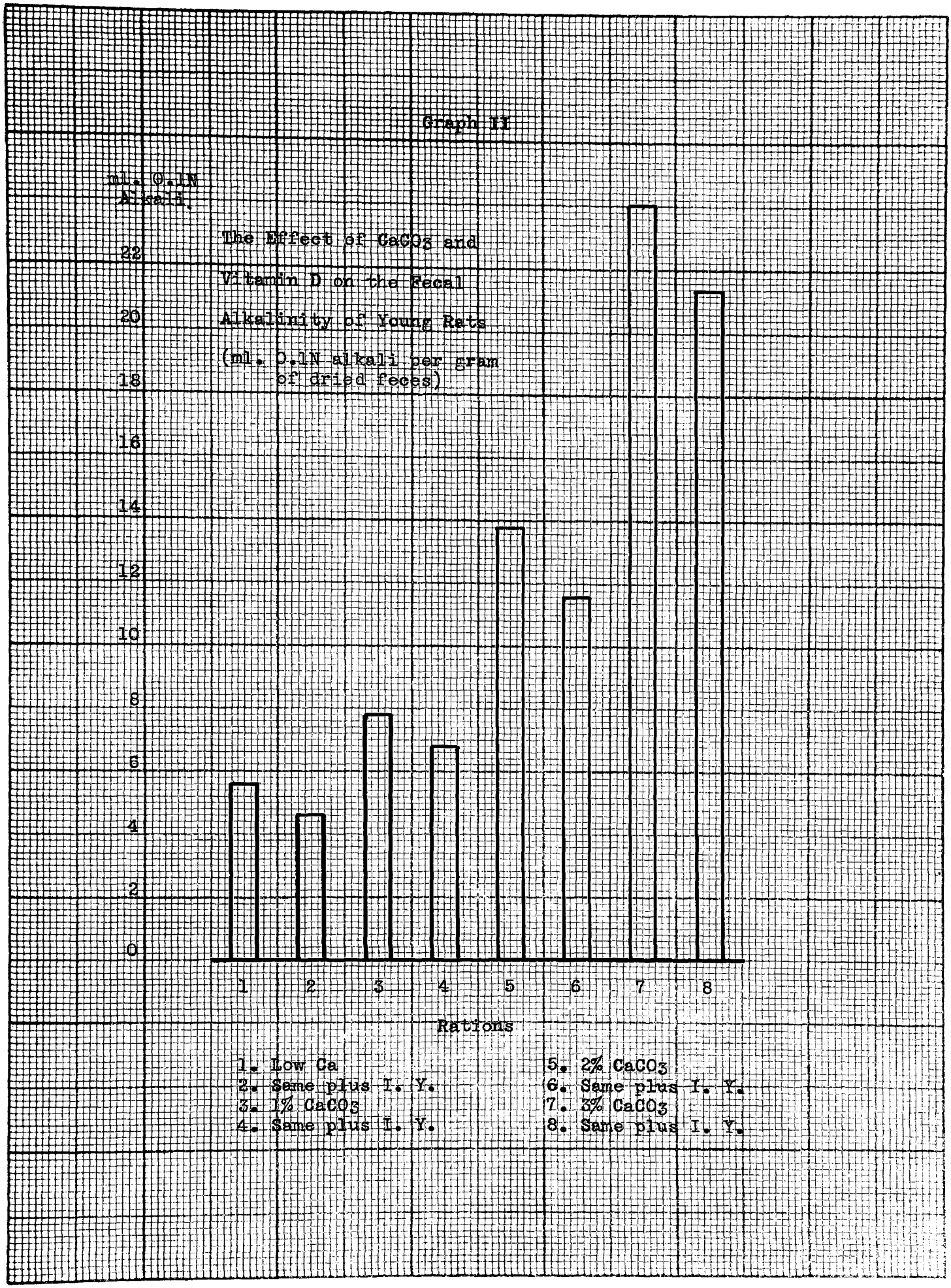
Table VII

Total Fecal Alkalinity of Young Rats Fed Various Rations with and without Vitamin D

(No. of ml. of 0.1N alkali per gram of dried feces)

Ration	1st week	2nd week	3rd week	4th week	Average
1 Low Ca ration	5.3	5.7	5.2	6.0	5.4
2 Same plus irrad. Y.	4.0	4.0	4.6	4.7	4.3
3 1% $CaCO_3$ ration	7.4	8.4	8.4	7.2	7.8
4 Same plus irrad. Y.	7.3	6.5	7.3	5.8	6.7
5 2% $CaCO_3$ ration	13.8	15.0	15.0	11.6	13.8
6 Same plus irrad. Y.	12.9	11.7	11.1	10.1	11.4
7 3% $CaCO_3$ ration	22.0	24.7	26.4	22.9	24.0
8 Same plus irrad. Y.	21.8	19.8	22.9	21.1	21.3

Graph II


ml. 0.1N Alkali.
The Effect of CaCO3 and Vitamin D on the Fecal Alkalinity of Young Rats
(ml. 0.1N alkali per gram of dried feces)
22
20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
Rations
1. Low Ca
2. Same plus I. Y.
3. 1% CaCO3
4. Same plus I. Y.
5. 2% CaCO3
6. Same plus I. Y.
7. 3% CaCO3
8. Same plus I. Y.

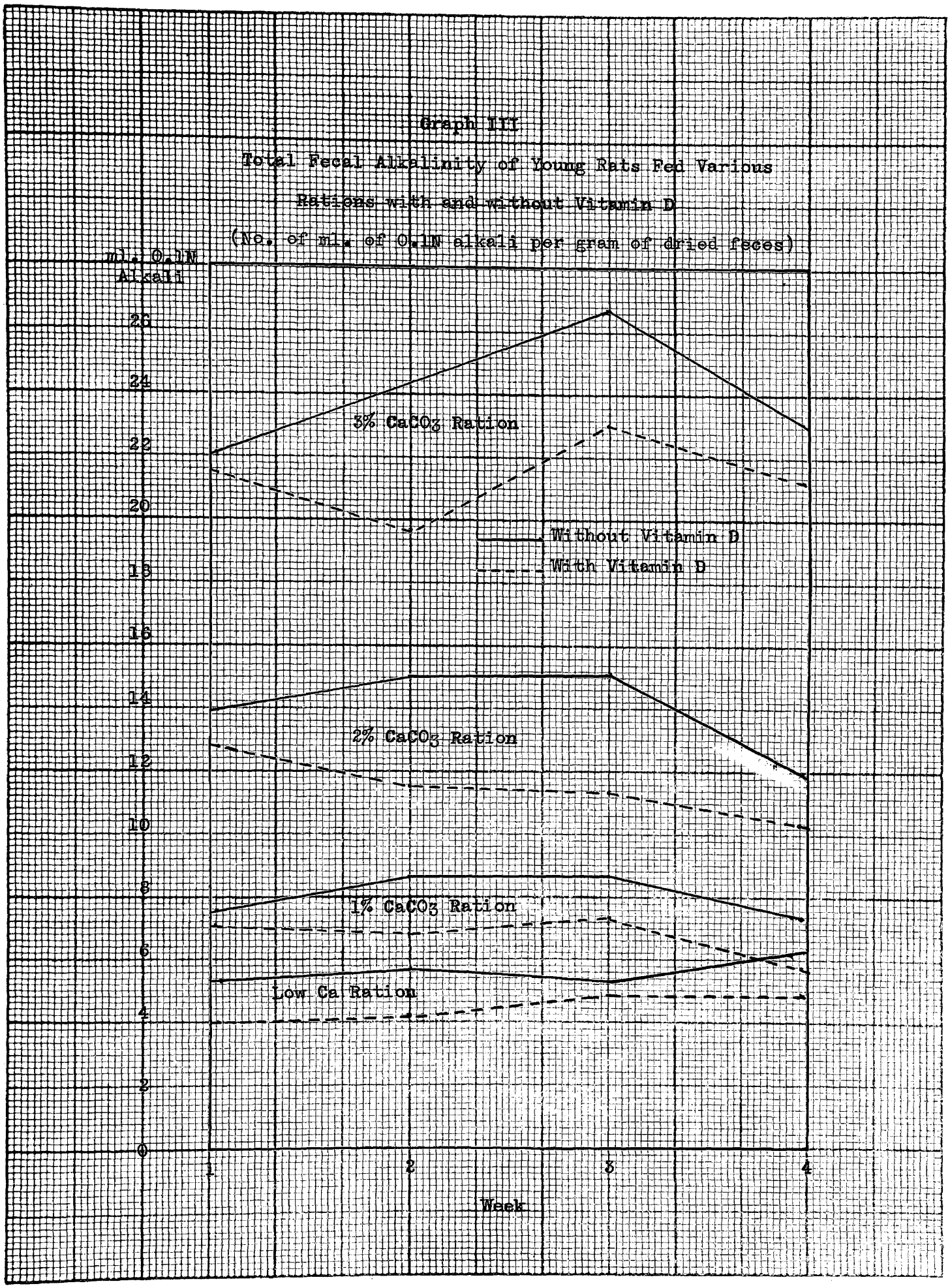

Graph III
Total Fecal Alkalinity of Young Rats Fed Various Rations with and without Vitamin D
(No. of ml. of 0.1N alkali per gram of dried feces)
ml. 0.1N Alkali
26
24
22
20
18
16
14
12
10
8
6
4
2
0
3% CaCO3 Ration
Without Vitamin D
With Vitamin D
2% CaCO3 Ration
1% CaCO3 Ration
Low Ca Ration
1
2
3
4
Week

Table VIII

Total Fecal Alkalinity of Adult Rats Fed Various Rations with and without Vitamin D

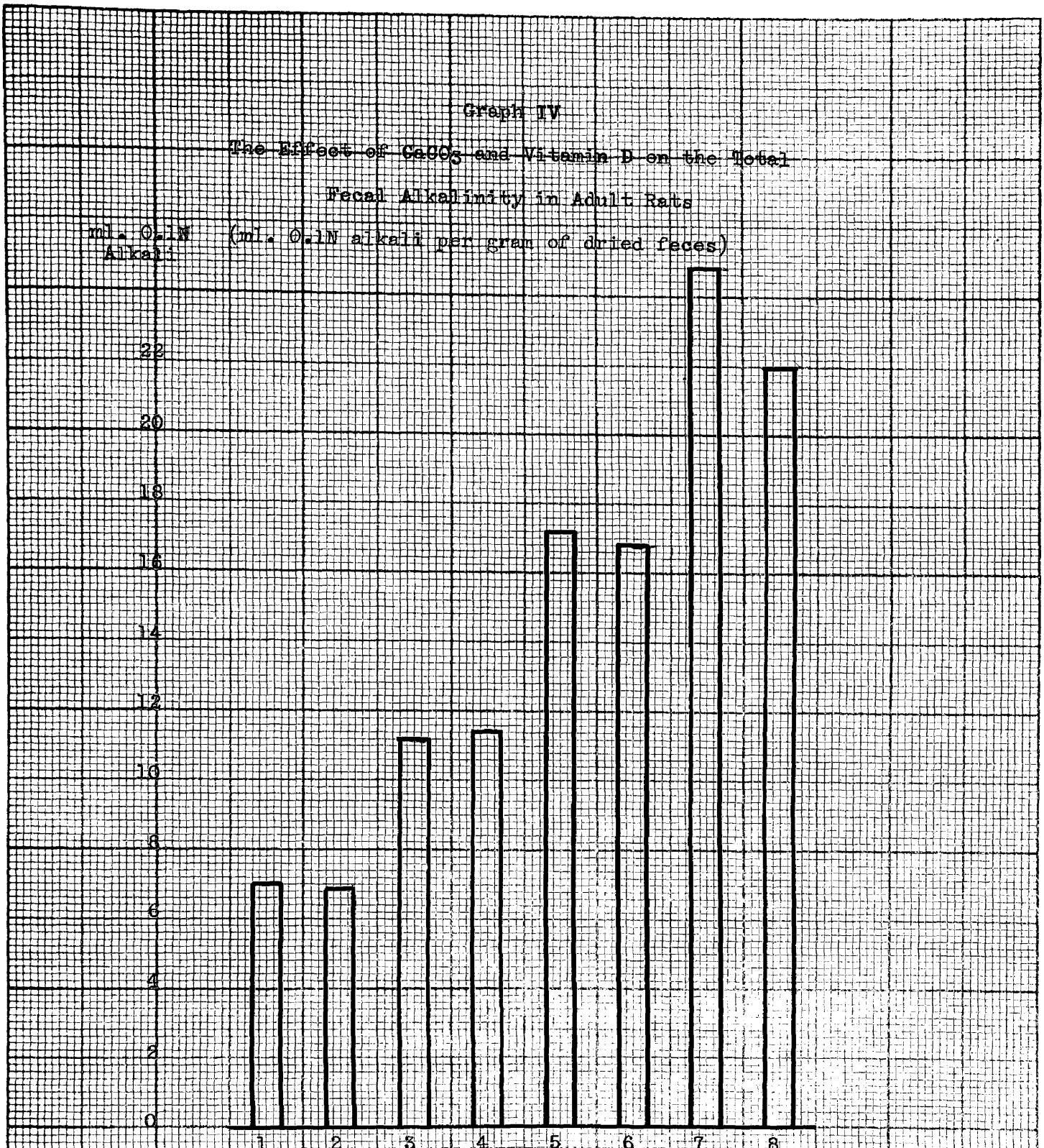
(No. of ml. of 0.1N alkali per gram of dried feces)

Ration	1st week	2nd week	3rd week	4th week	Average
1 Low Ca ration	8.2	7.8	6.5	6.7	7.3
2 Same plus irrad. Y.	7.8	7.1	6.5	6.3	6.9
3 1% $CaCO_3$ ration	13.6	9.8	9.8	10.3	10.9
4 Same plus irrad. Y.	13.2	10.6	11.5	10.9	11.5
5 2% $CaCO_3$ ration	18.1	17.1	17.7	16.3	17.3
6 Same plus irrad. Y.	18.1	17.1	16.4	16.3	17.0
7 3% $CaCO_3$ ration	17.7	26.2	28.6	30.2	25.6
8 Same plus irrad. Y.	17.7	22.5	23.1	26.0	22.3

Graph IV

The Effect of $CaCO_3$ and Vitamin D on the Total Fecal Alkallinity in Adult Rats

ml. 0.1N Alkali (ml. 0.1N alkali per gram of dried feces)



1. Low Ca
2. Same plus I. V.
3. 1% $CaCO_3$
4. Same plus I. V.
5. 2% $CaCO_3$
6. Same plus I. V.
7. 3% $CaCO_3$
8. Same plus I. V.

C. THE EFFECT OF RICKETS AND VITAMIN D ON PLASMA PHOSPHATASE ACTIVITY IN RATS

HISTORICAL

In 1932 Kay published an extensive review on the subject, "Phosphatase in Growth and Disease of Bone". For our purpose only the literature dealing directly with the effect of rickets and vitamin D on plasma phosphatase activity will be given. Working with children Bodansky and Jaffe (1934) found that serum phosphatase activity was an excellent criterion of the severity and rate of healing of rickets. Kinard and Chanutin (1933) concluded that irradiated ergosterol, while causing a rise in phosphatase values with growing rats, has no effect on adult rats. Common (1936) observed a marked increase in blood phosphatase values in young chicks but noted wide variations of the values of the enzyme in laying hens as well as in cocks. He also found that the development of rickets in chicks produced a very marked increase in the phosphatase activity level of the serum. Mitchell (1936), studying the serum phosphatase in 75 cases of fractures in adults, failed to find any significant changes in serum phosphatase following fracture. He then concluded that the serum phosphatase value was not an index of healing or of the rate of healing of the fractures. Crinim and

Strayer (1936) found a reduction in blood phosphatase value in rats only after the administration of toxic doses of vitamin D in the form of viosterol. There seems to be no comprehensive study reported in the literature correlating vitamin D and rickets with plasma phosphatase activity in rats. The aim of the present investigation was to see whether or not vitamin D had any definite effect on the phosphatase activity in rats by studying the problem extensively under various experimental conditions.

EXPERIMENTAL

Jenner and Kay's method (1932) was followed throughout this experiment. The animals were killed by etherization, and blood samples were obtained immediately by puncturing the heart. The phosphatase content of a sample of plasma is defined as the number of milligrams of P which would be liberated by 100 ml. of the plasma under the conditions perscribed.

Since no consistant results were obtained in several preliminary experiments on rats of varying ages fed both normal and rachitogenic diets and rachitogenic diets supplemented with vitamin D, it was decided to plan a more comprehensive study on this problem. Three series of experiments were included in this study. In Series I, both young and adult normal rats were tested. These animals were taken from the breeding stock which had been fed a ration consisting

of: maize, 30; oats, 30; milk powder, 30; linseed meal, 6, ground alfalfa, 5; yeast, 3 and NaCl, 1. Animals in Series II were given a high Ca rachitogenic diet containing maize, 50; oats, 25; wheat gluten, 20; $CaCO_3$, 3 and NaCl, 1. All animals in this group were placed on the rachitogenic diet when they were approximately 21-25 days of age, weighing between 50 and 55 grams. Those receiving the basal diet alone were killed at the end of 31 days, the usual testing period for rachitic rats. Vitamin D supplements in the form of irradiated yeast were added 21 days after the animals had been on the rachitogenic diet. Animals were killed for plasma P and phosphatase activity determinations at day intervals after feeding the supplements. A low Ca rachitogenic diet was employed in Series III. This was the same as the high Ca diet used in Series I with the exception that $CaCO_3$ was omitted. Various modifications in diet were made at the end of 21 days and all animals were killed 10 days later.

RESULTS AND DISCUSSION

Complete results are presented in Table IX.

No appreciable differences were observed in plasma phosphatase activity between high Ca and low Ca rickets, and between rachitic animals and rachitic animals given a supplement of vitamin D. In general, the phosphatase activity values were inconsistant and

duplicate or repeated determinations within any given group varied considerably. From these results it appeared clear that at least, in rats, the plasma phosphatase activity can not be employed to indicate the severity of rickets or the degree of healing of rickets.

It is generally believed that rickets experimentally produced in rats is of an entirely different nature as compared with the rickets found in children. In rats the rachitic condition is produced not primarily by depriving the animal of vitamin D but chiefly by upsetting the Ca:P ratio in the diet. Besides, there is a decided difference in the requirement of vitamin D between the rat and the child. In children, even under normal conditions a certain amount of vitamin D must be available constantly in the system, in order to assure proper bone development and to prevent rickets while in rats, unless the Ca-P metabolism is greatly disturbed such as in the production of experimental rickets, little or no vitamin D is needed for normal calcification. Under such conditions it appears doubtful that any comparable results can be obtained in the study of the plasma phosphatase activity of the rat and that of the child.

Furthermore the phosphatase activity of the rat has been found to be at a peak during the early stages of life at which time bone development is proceeding at its greatest speed. Kinard and Chanutin (1933) reported that the phosphatase activity of the whole rat reaches a maximum between the 13th and the 20th day of age;

thereafter there is a decrease until, at the 60th day the concentration of the enzyme remains constant. Klarin (1935) obtained the maximum values at the end of the first week, a decrease followed in a few days and at the end of 6 to 8 weeks it was low and fairly constant. All this evidence points to the conclusion that at the age when experimental rickets is produced in rats (6-7 weeks old) the animals have passed the stage during which body phosphatase was greatest, hence the enzyme is not readily affected either by the production of rickets or by the action of vitamin D. This may help to explain why the results obtained in the study of plasma phosphatase activity are of no particular significance or are at variance with studies made on infants.

Table IX

Vitamin D and Plasma Phosphatase Activity in Rats

Animal and Experimental Conditions	No. Animals Used	Plasma P	Phosphatase Activity (Ave. Values)
SERIES I			
9 week old young normal rats	2	6.8	39.42
	2	6.7	32.94
	2	5.8	24.84
	2	6.0	19.38
Normal young rats starved 24 hours	2	6.35	18.90
Normal adult rats	4	4.75	11.52
SERIES II			
High Ca rickets	2	2.6	32.40
	2	2.25	26.88
High Ca rickets supplemented with 0.01 gm irrad. yeast			
Killed at 2 days	4	3.25	33.24
Killed at 4 days	4	3.4	34.56
High Ca rickets supplemented with 0.1 gm irrad. yeast			
Killed at 2 days	4	3.9	32.16
Killed at 4 days	4	4.0	37.56
Killed at 5 days	4	4.1	30.78
Killed at 10 days	4	5.25	34.02
High Ca rickets supplemented with 0.5 gm irrad. yeast			
Killed at 5 days	4	5.55	30.12
High Ca rickets supplemented 1 gm irrad. yeast			
Killed at 2 days	2	4.5	34.38
Killed at 4 days	2	4.6	44.10
SERIES III			
Low Ca rickets	4	6.10	38.76
	2	5.10	37.50
Low Ca rickets supplemented with 0.1 gm irrad. yeast	4	4.9	40.20
Low Ca rickets supplemented with: stock ration	4	6.1	41.82
1% $CaCO_3$ ration	4	4.7	27.90
3% $CaCO_3$ ration	4	3.4	23.82
irradiation, 1 min.	4	3.3	35.94

SUMMARY AND CONCLUSIONS

AND

BIBLIOGRAPHY

SUMMARY AND CONCLUSIONS

1. Some studies have been made on the production of rickets in rats with special reference to the rachitogenic diets. By substituting granulated corn for ordinary corn in the Steenbock ration 2965 with the addition of 25% oatmeal and 1% dried yeast a very satisfactory rachitogenic ration was obtained. This modified ration was found to give better results than the Steenbock ration 2965 when employed for ordinary vitamin D assay in the degree of rickets produced, the growth of the test animals during the experimental period and the general appearance of the lines in the line test.

2. A preliminary study has been conducted in the vitamin D content of irradiated and sun-cured corn. Corn irradiated for one minute under an ultra violet lamp was found to be highly antirachitic, while the sun-cured corn (exposed to October sunlight for 10 hours) contained a small but significant amount of the antirachitic factor. The exposure of corn to sunlight of greater ultra violet intensity might easily result in the production of sufficient vitamin D to seriously reduce the rachitogenic properties of a diet.

3. The mode of action of vitamin D has been studied from the standpoint of its possible effect on the total fecal alkalinity and the plasma phosphatase activity in rats. The total alkalinity of the feces of the test animals was found to be directly proportional to the amount of $CaCO_3$ included in the ration.

When vitamin D was added to the ration the alkalinity was reduced in each case. This finding seemed to confirm the "net absorption" or "net retention" theory of Harris.

4. No appreciable differences were observed in plasma phosphatase activity between rachitic rats and rachitic rats receiving a supplement of vitamin D. In rats, the plasma phosphatase activity can not be employed to indicate the severity of or the degree of healing of rickets.

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