AN ABSTRACT OF THE THESIS OF

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Title _A Study of Certain Effects Produced in Guinea Pigs_

Deficient in Anti-Stiffness Factor

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Abstract Approved: Redacted for privacy Redacted for privacy
                        (Major Professor)

This investigation was based on the discovery by Wulzen and
Bahrs of a dietary disease which developed in guinea pigs maintained
on a basal diet of skimmed milk. The disease in the animals was
characterized by development of stiffness at the joints followed by
degenerative changes in the tissue and abnormal deposits of calcium.
The missing factor was called "anti-stiffness" factor. Van Wagtendonk
has isolated a factor from raw cream which was capable of relieving
the stiffness. This factor was called curative factor in this study.

The object of the study was: 1, to determine the urinary
excretion of phosphorus in the normal guinea pigs and the effect of
the curative factor on the urinary phosphorus excretion; 2, to
ascertain whether the curative factor added to the deficient diet
would prevent the development of the stiffness and abnormal calcifi-
cation.

Part I

Urinary phosphorus determination. The molybdate method of
Youngsberg and Youngsberg and Fiske and Subbarrow was used for
determining phosphorus in twenty-four hour samples of urine.

The urinary phosphorus was determined for forty-six normal
animals and was found to be low.

Urinary phosphorus levels were determined on one hundred
twenty-six guinea pigs on the diet deficient in the "anti-stiffness"
factor, and it was found that the phosphorus excretion was much
higher in these animals than in the normal animals.

Urinary phosphorus determinations were made for one hundred one
guinea pigs; these animals were fed the deficient diet and treated
with various amounts of the curative factor. When Wesson oil was
given to ten animals for five days, it was found that the urinary
phosphorus level was unchanged. Large doses of the curative

factor (10 gamma) were given to twenty-eight guinea pigs for five days; the phosphorus excretion was lower at the end of treatment than before treatment started. The urinary phosphorus level increased in the thirty-three animals which were given small doses of the curative factor (0.01 and 0.1 gamma) for seven to fourteen days.

It was concluded that 1, the normal guinea pigs (controls) had a normal phosphorus excretion; 2, the animals on the diet deficient in "anti-stiffness" factor had a high urinary phosphorus excretion; 3, the urinary phosphorus in guinea pigs on the deficient diet was changed when the animals were treated with the curative factor. If a large dose of curative factor was given, the phosphorus level decreased. If a small dose of curative factor was given, the urinary phosphorus level increased. The results were based on a short time of treatment.

Part II

Thirty guinea pigs were fed on the diet deficient in "anti-stiffness" factor. From the time they were started on the deficient diet eight of the animals were given a supplement of 1.5 gamma of the curative factor.

The eight animals which were given the supplement remained normal in appearance and never developed the typical stiffness in the wrist joints; the other twenty-two animals developed the stiffness in varying degrees.

Four animals from each set were killed for autopsy and compared carefully. The tissues and organs were perfectly normal in the four guinea pigs which had received the supplement. Some type of tissue degeneration or calcification was seen in each of the four animals which received no supplement.

The skeletons of these animals were compared but little difference was observed except a tendency for the limb bones to be somewhat shorter in the animals which were given the curative factor.

It was concluded that animals on the diet deficient in the "anti-stiffness" factor can be protected from the usual symptoms by giving small doses of the curative factor.

CERTAIN EFFECTS PRODUCED IN GUINEA PIGS
DEFICIENT IN AN ANTI-STIFFNESS FACTOR

by

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A THESIS

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CERTAIN EFFECTS PRODUCED IN GUINEA PIGS
DEFICIENT IN AN ANTI-STIFFNESS FACTOR

INTRODUCTION

Literature Review. Phosphorus metabolism has been
very extensively studied, especially as it is related to
calcium metabolism.

The most recent development for the study of mineral
metabolism has been the application of isotopes as indi-
cators (Greenberg and Gunther)[10]. Induced radio active
isotopes may be traced in the organism until they are
excreted. Radio active phosphorus is easily prepared, has
a relatively long half life, and gives off fairly intense
radiations, making its utilization practicable. By the use
of radio active phosphorus Hevesy and collaborators were
able to demonstrate that radio active phosphorus reached
the teeth, muscles, bones and other organs (Greenberg)[9].

Phosphorus may be stored as calcium salts in bone but
most of the phosphorus is in the soft tissues (Schmidt and
Greenberg)[21]. Phosphorus always occurs as derivatives of
phosphoric acid; it may be in the form of inorganic salts,
lecithin or phospholopids, nucleic acids, and nucleo
proteins (Schmidt and Greenberg)[21]. The phosphorus com-
pounds that may be in the blood are: 1, inorganic phosphate;
2, nucleotides; 3, glycerophosphoric acid; 4, organic phos-
phate esters; 5, phospholipids; 6, absorable calcium-

phosphorus complex (Benjamin and Hess[4]; Schmidt and Green-
berg[21]). The absorbable calcium-phosphorus complex com-
prises from 50-75 per cent of the total serum phosphorus
(Benjamin and Hess)[4]. The blood level of inorganic phos-
phorus of adult animals usually ranges from 0.5 mg. to
3.4 mg. per cent, varying with the species (Benjamin and
Hess)[4].

The level of the phosphorus excretion is controlled
largely by the phosphorus intake. A large proportion of
the urinary phosphorus is inorganic, but there is ordin-
arily a small amount of organic phosphorus present (Brull[5];
Hawk and Bergeim[13]; and Walker[24]). Walker[24] found that the
excretory rate of inorganic phosphorus in normal individu-
als had a rather wide range (4.44 mg--63.7 mg per hour).

It is recognized that a threshold of plasma phosphorus
acts as a controlling factor in urinary excretion of phos-
phorus. The ordinary threshold is 2.0 to 2.4 mg. P per
100 c.c. of whole blood, this level being maintained by
parathyroid and hypophysis secretions (Brull[5]; Walker[24]).
Brull[5] suggests that renal mechanism probably plays a part
in maintaining the threshold. The normal concentration of
soluble calcium and phosphate ions in the blood plasma is
probably as high as possible without initiating precipita-
tion of the calcium-phosphorus salts. The maximum balances
are physiologically controlled by absorption and excretion
processes (Logan)[17].

Vitamin D is another controlling factor for phosphorus levels in the organism. At present most workers agree that the development of rickets is associated with a decrease, first of inorganic phosphorus (Greenberg[9] and Youmanns[28]) and adenosintriphosphate in the blood serum, and then of diphospho-glyceric acid in the corpuscles. (Rapoport and Guest)[18] Benjamin and Hess[4] feel that the decrease of the absorbable calcium-phosphorus complex in the blood of rachitic animals is more dependable and more often manifest. Excess vitamin D produces a rise in blood phosphorus (Duncan and Huffman[7]; Ashford[1]; Reed, Struck, and Steck[19]; Hess and Lewis[14]; Harris and Moore[12]; and Greenberg[9]). There is diverse opinion regarding the effect produced by an excess of vitamin D (hypervitaminosis D) on urinary excretion of phosphorus and calcium. Reed and co-workers[19] regarded vitamin D as being capable of producing random variations of urinary calcium and phosphorus. Gough, Duiguid, and Davies[11] maintained that there is a reciprocal relation between the level of phosphorus and calcium in urinary excretion, an increase of one being accompanied by a drop in the other. Ashford[1]; Bauer[3]; and Duncan and Huffman[7] reported that excess doses of vitamin D tend to raise the urinary calcium and phosphorus.

The mode of action of vitamin D is not yet clearly understood. It may regulate absorption of calcium and phosphorus from the gut (Ashford[1]; and Kern, Montgomery and

Still[15]). On reviewing the literature on the subject Schmidt and Greenberg[21] were inclined to support the hypothesis that vitamin D functions in calcium and phosphorus deposition in bone rather than absorption from the gut. Schmidt and co-authors[21] recognized the fact that vitamin D increases the permeability of the intestinal wall to calcium and phosphorus but were inclined to think that other factors undoubtedly are involved, one of the most likely factors being the action of the phosphorus-ester splitting enzymes found in the digestive tract.

Gough, Duiguid and Davies[11]; Schulz[22] made studies on urinary phosphorus excretion in patients suffering from nephrosis. When normal and nephritic patients were given injection of acid sodium phosphate (NaH_2PO_4) or tertiary phosphate (Na_3PO_4), fifty per cent more phosphorus was recovered from the normal patients than from those with nephrosis. The authors felt that renal injury lessens phosphorus excretion. It was also observed that excretion and deposition of calcium was greatest when tertiary phosphate was given and that urinary phosphate was greatest when acid phosphorus was given.

Some hormones are known to affect the level of calcium and phosphorus. Parathyroid secretions (Cohn, Cohn and Aub[6]; and Greenberg[9]) have a solvent effect on bone, producing an increased phosphorus excretion, elevating serum calcium and depressing phosphorus concentration in serum.

Ovariectomy is reported to increase serum calcium concentration (Greenberg)[9].

In early investigations Bahrs and Wulzen[2, 25] found that when planarian worms were fed tissues from guinea pigs maintained on supposedly adequate diets, the worms developed a dietary disease. It was found that a protective factor against this deficiency was present in kale. The factor was called "pl". This work led to the development of a basal diet of skimmed milk which produced in guinea pigs a definite deficiency disease (Wulzen)[26]. The characteristic condition which developed was a pronounced stiffness at the joints of limbs, followed by degenerative changes in the tissues and the appearance of abnormal deposits of calcium in various organs. The missing factor has been termed "anti-stiffness" factor. It was found that raw cream had a preventive and curative action. Van Wagtendonk[23] has isolated a factor from raw cream which is capable of relieving the stiffness; this factor has been called curative factor in this paper.

The abnormal deposition of calcium in the animals deficient in the "anti-stiffness" factor suggests that calcium and phosphorus metabolism has been disturbed. In order to explain the possible changes that take place, studies of the various stages of calcium and phosphorus metabolism appear to be necessary.

The purpose of the investigation was to:

1. Determine the urinary excretion of phosphorus in normal and deficient guinea pigs and the effect of the curative factor on the urinary phosphorus excretion.

2. Ascertain whether the curative factor added to the deficient diet would prevent the development of the stiffness and abnormal calcification.

PART I

EXPERIMENTAL

Urinary phosphorus excretion was determined on the following groups of animals: Group I, normal guinea pigs (Controls), Group II, guinea pigs deficient in the "anti-stiffness" factor, and Group III, deficient guinea pigs treated with the curative factor.

The normal animals were maintained on a diet of kale, barley, straw, and iodized salt ad. lib.

The animals in Groups II and III were fed a basal diet containing an eighteen per cent mixture of skimmed milk powder in water with 0.78 mg. of copper sulfate and 4.82 mg. ferric chloride added to each 100 ml. of the mixture. To the above mixture was added a daily supplement of vitamins in the following quantities for each animal: Water soluble vitamins (Pyridoxin hydrochloride 0.1 mg., nicotinic acid 1.0 mg.), thiamin HCl 0.2 mg., riboflavin 0.5 mg., pantothenic acid 0.1 mg., inositol 10.0 mg., p-aminobenzoic acid 2.0 mg., choline 50.0 mg., and biotin 0.01 mg.; fat soluble vitamins, Beta carotene 150 I. U.; viosterol 40 I. U.; alpha-tocopherol 0.1 mg.; and 2-CH$_3$-, 1-4, naphthoquinone 0.1 mg. Four milligrams of ascorbic acid per animal was fed orally on alternate days. The animals were given autoclaved straw and salt ad. lib.

The animals on the above diet developed the syndrome described by Wulzen[26]. The degree of stiffness which developed in the wrist was determined by pulling the forelimb of the animal back parallel to the body. The limb was held up with the fingers while the thumb pressed down firmly on the olecranon process. Under these conditions the joint of a normal animal will easily bend to a position forming a right angle with the leg. If the joint of the animal had been affected, the foot could not be bent to a right angle position.

Phosphorus analysis was made on twenty-four hour samples of urine collected from animals placed in metabolism cages. Except for early determinations, all samples of urine were diluted to 100 ml. or 250 ml. if more than 100 ml. were excreted.

The procedure used was somewhat modified from the methods of Youngsberg and Youngsberg[28] and Fiske and Subbarow[8]. The principle of the reaction is the formation of phosphomolybdate subsequently reduced with 1-amino-2-naphthol sulfonic acid to a blue compound.

One milliliter of urine was diluted to 100 ml; to two ml. of this dilution 6.6 ml. of water, 2 ml. of molybdate reagent (50 ml. of 7.5 per cent ammonium molybdate diluted to 100 ml. with 10N H_2SO_4), and 0.4 ml. of 1-amino-naphthol sulfonic acid were added. After standing for 10 minutes the color was read in a Klett colorimeter, using a filter

with a 640-700 millimicron spectral range.

The total P was determined by wet combustion of 1 ml. of urine with 6 ml. of 10N H_2SO_4, 5-10 drops of concentrated HNO_3 and 5-10 ml. of water; this was boiled for 20 minutes to insure complete combustion. The reaction mixture was diluted to 100 ml. and two milliliters of the solution used for the determination of phosphorus (50 ml. of 7.5 per cent molybdate solution diluted to 100 ml. with 25 ml. of 10N H_2SO_4 and 25 ml. of 10N H_2SO_4).

The color which developed for the inorganic and total phosphorus was compared with that produced by a standard phosphorus solution containing 0.005 mg. P/ml.

Total and inorganic phosphorus were calculated as milligrams of phosphorus per kilogram body weight. The following equation was used.

$$P = \frac{0.01 \text{ X Reading of Unknown X 100 gm. X vol. of urine X 50}}{\text{Reading of Standard X Weight of animal}}$$

Organic phosphorus was calculated as the difference between total phosphorus and inorganic phosphorus.

The significant difference between groups was calculated according to the following formula.

$$\sigma = \sqrt{\frac{\Sigma d^2}{N}}$$

$$S.E. = \frac{\sigma}{\sqrt{N}}$$

$$S.D. = \frac{mean_1 - mean_2}{\sqrt{S.E._1 + S.E._2}}$$

σ Standard deviation
Σ Sum
d   deviation
N   number of individual determinations
S.E. Standard error between the mean and the deviation of individual determinations

S.D. Significant difference between two groups, sig-
nificant if > than 2.

All determinations are summarized in Tables I, II, III,
and IV.

Group I (Controls). The inorganic and organic phos-
phorus were determined in the urine of forty-six normal
animals. The results are shown in Table I.

Group II (Deficient in "anti-stiffness" factor). Phos-
phorus in the urine was determined on thirty animals that
had been on the diet for fifteen months and on ninety-six
animals which had been on the diet for two to six months.
Table I shows the level of inorganic and organic phosphorus
for this group and the significant difference when compared
to Group I.

Group III (Deficient in "anti-stiffness" factor;
treated with the curative factor). This group was divided
into ten subgroups, according to the amount of curative fac-
tor given. With the exception of subgroups E and H, the
phosphorus was determined on the day before the treatment
was started (deficient day) and on the last day of treatment
with the curative factor. The significant difference was
calculated for the change in phosphorus level from the first
determination (deficient day) to the last day of treatment.

Subgroup A. Ten animals were given 0.5 ml. of Wesson
oil per day for five days; urinary phosphorus was determined
on the deficient day and the last day of treatment. See
Table II.

Subgroup B. Ten animals were given a 0.1 gamma dose of curative factor per day for eight days. The analyses of phosphorus were made on the deficient day and the eighth day of treatment. Results for this group are given in Table II.

Subgroup C. Ten animals which had been on the deficient diet for 6 months were given 1.0 gamma of the curative factor per day for seven days. Determinations of the urinary phosphorus levels were made on the deficient day and the seventh day of treatment. The results are shown in Table II.

Subgroup D. Ten animals which had been on the diet for 2 months were treated as in subgroup C; results are given in Table II.

Subgroup E. Five animals were treated five days with 0.01 gamma dose of factor. Phosphorus analyses were made on the second and fourth day of treatment. See Table III.

Subgroup F. Eight animals were given 0.01 gamma per day of curative factor for seven days. Phosphorus determinations were made on the deficient day and the seventh day of treatment. Results are given in Table II.

Subgroup G. Ten animals were given 0.01 gamma per day of the curative factor for fourteen days; phosphorus determinations were made on the deficient day and the fourteenth day of treatment. See Table II.

Subgroups H-K. The urinary phosphorus excretion is shown by a graph in Table IV. The initial and final phosphorus levels are indicated at the end of each curve on the

graph; the top line represents the total phosphorus and the
bottom line the inorganic phosphorus, and the difference
between the two levels represents the organic phosphorus.
Subgroups H, I. and J. Ten animals were used in each group;
they were given 10 gamma of the curative factor per day for
five days. Subgroup K. Eight animals were given ten gamma
of the curative factor per day for seven days.

In the tables the following abbreviations are used:

    S.E.   standard error
    S.D.   significant difference
    Sg.   Subgroup

TABLE I

Urinary Phosphorus Levels for Normal (Controls) and Deficient Animals

	Group	Number of Animals	Time on Diet	Inorg. P. mg/kg/24hr	Total P. mg/kg/24hr	Org. P. mg/kg/24hr	S.D. I.P.	S.D. T.P.
	Controls	46	None	15.2	20.8	5.6		
S.E.				1.3 $\pm$	1.6 $\pm$			
	Deficient	30	15 mo.	63.1	71.8	8.7	19.1	11.1
S.E.				4.7 $\pm$	5.2 $\pm$			
	Deficient	96	2-7 mo.	79.0	85.6	6.6	29.4	28.9
S.E.				3.4 $\pm$	3.4 $\pm$			

TABLE II

Urinary Phosphorus Levels for Subgroups A, B, C, and D
Amount of Curative Factor Given and Number of Days Treated Shown in Column 2

	Group	Number of Animals	Time on Diet	Deficient Inorg. P. mg/kg/24hr	Total P. mg/kg/24hr	Org. P. mg/kg/24hr	Last Day Inorg. P. mg/kg/24hr	Total P. mg/kg/24hr	Org. P. mg/kg/24hr	S.D. I.P.	S.D. T.P.
	A 0.0	10	6 mo.	84.5	93.7	9.2	78.3	96.4	18.1	1.6	0.7
S.E.	5 days			8.0 $\pm$	8.9 $\pm$		5.9 $\pm$	5.6 $\pm$			
	B 0.1	10	7 mo.	61.6	83.9	22.3	87.5	96.9	9.4	5.9	2.7
S.E.	8 days			7.6 $\pm$	10.7 $\pm$		10.4 $\pm$	12.3 $\pm$			
	C 1	10	6 mo.	67.8	87.8	10.0	75.7	79.9	4.2	2.0	2.1
S.E.	7 days			9.6 $\pm$	7.9 $\pm$		6.1 $\pm$	6.7 $\pm$			
	D 1	10	2 mo.	115.6	123.3	7.7	95.8	102.6	6.8	4.0	4.5
S.E.	7 days			15.2 $\pm$	14.2 $\pm$		9.1 $\pm$	7.3 $\pm$			

TABLE III

Deficient Animals Treated with 0.01 Gamma of Curative Factor
(Determinations for Subgroup E on Second and Fourth Day of Treatment)

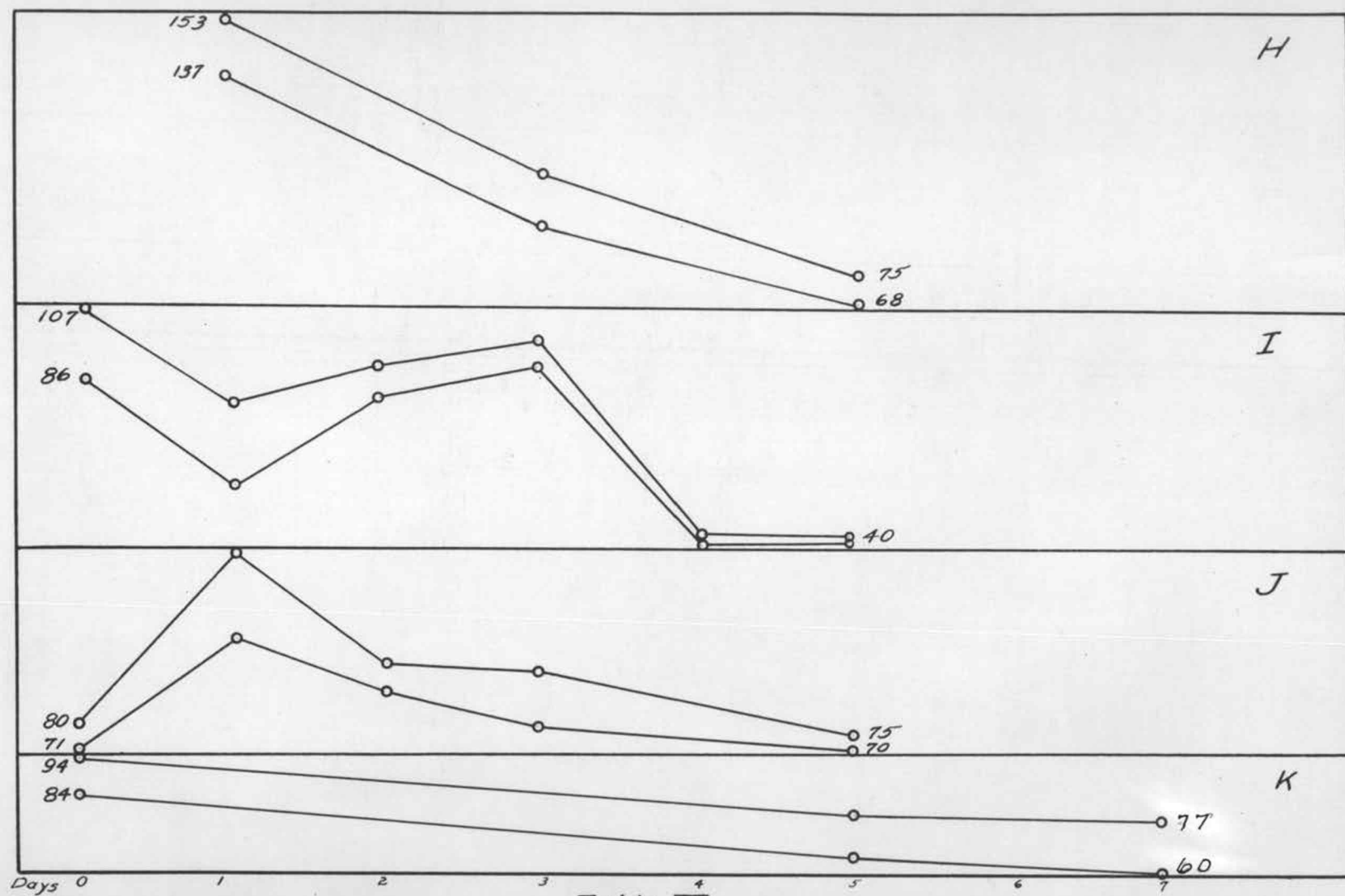| Group	Number of Animals	Time on Diet	Deficient			Last Day			S.D.	
			Inorg. P. mg/kg/24hr	Total P. mg/kg/24hr	Org. P. mg/kg/24hr	Inorg. P. mg/kg/24hr	Total P. mg/kg/24hr	Org. P. mg/kg/24hr	I.P.	T.P.
Sg. E 5 days	5	4.5 mo.	78.6	83.8	5.2	79.9	93.5	9.6	0.02	2.0
S.E.			12.1$\pm$	9.4$\pm$		13.0$\pm$	7.3$\pm$			
Sg. F 7 days	8	5.5 mo.	87.4	97.8	10.4	102.9	118.9	16.0	3.9	4.4
S.E.			7.9$\pm$	11.4$\pm$		7.6$\pm$	11.3$\pm$			
Sg. G 14 days	10	5 mo.	58.9	70.2	11.3	70.9	79.5	8.6	3.4	2.6
S.E.			6.3$\pm$	5.1$\pm$		5.6$\pm$	7.3$\pm$			

Table IV

Figures: H, I, J, and K for subgroups H, I, J, and K   10 Y Treatment

RESULTS

The results for the forty-six normal animals and the one hundred twenty-six deficient guinea pigs (Group I and Group II) are given in Table I. The normal animals had a low phosphorus excretion in the urine. The deficient animals were found to have a phosphorus excretion which was significantly higher than the normal animals. The phosphorus excretion for the animals which had been on the deficient diet for 2-7 months was much higher than for the animals which had been on the diet for 15 months. The organic phosphorus excretion for the normal and the deficient animals was nearly the same.

Tables II, III, and IV show the results for the guinea pigs deficient in the "anti-stiffness" factor when various amounts of the curative factor were given (Group III). The inorganic and organic phosphorus excretion were determined on one hundred one animals given the factor. The following changes were observed.

Table II. The urinary phosphorus levels were unchanged for the ten animals in subgroup A which were given Wesson oil for five days. The ten animals which were given 0.1 gamma of the curative factor for eight days, had an increased phosphorus excretion at the end of the treatment. When subgroup C (on the diet six months) and subgroup D (on the diet two months) were treated seven days with

1 gamma of the curative factor, Group C showed no change in urinary phosphorus excretion while subgroup D showed a lowering in phosphorus excretion.

Table III. There was an increase in the phosphorus excretion for the twenty-three animals which were given the 0.01 gamma of the curative factor. The increase of the urinary phosphorus was not significant for the five animals whose excretion was determined on the second and fourth day of treatment. For the eight animals in subgroup F and the ten animals in subgroup E the urinary phosphorus excretion was higher on the last day of treatment with the factor.

Table IV. The urinary phosphorus levels are shown graphically for the thirty-eight animals which were treated with 10 gamma of the curative factor per day. The phosphorus levels on the last day of treatment were lower than at the initial determination, the deficient day. The significant difference for each group was

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Subgroup H   T.P.  19.1
             I.P.  17.6
Subgroup I   T.P.  18.1
             I.P.  11.4
Subgroup J   T.P.   1.0
             I.P.   0.1
Subgroup K   T.P.   4.3
             I.P.   5.6
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There was a significant decrease in urinary phosphorus excretion for subgroups H, I, and K. Animals in subgroup J did not eat the diet well while in the metabolism cages which may explain why the phosphorus level was only slightly changed.

The organic phosphorus in the urine was variable for the deficient animals treated with the curative factor; there was a decrease in the level of organic phosphorus for subgroups C, B, G, H, I, and J, and an increase for subgroups D, E, G, and K.

DISCUSSION

The data in Table I shows that normal animals (Group I, controls) have a low phosphorus excretion which correlates with the relatively low intake (about 450 mg./100 grams of food). The animals on the diet deficient in the "anti-stiffness" factor (Group II) were maintained on a diet with a relatively high phosphorus content (about 960 mg. P/100 gm. of food); this partially explains the high phosphorus excretion. The fact that the animals which had been on the diet for a short time had a much higher urinary phosphorus excretion level than those which had been on the diet for a much longer time indicates that the deficiency produces some change in the organism which affects the amount of phosphorus excretion in the urine. The absence of the "anti-stiffness" factor may affect the processes of absorption, deposition or excretion of phosphorus in the organism and thus cause the changes in urinary excretion.

When the animals on the diet deficient in the "anti-stiffness" factor were treated with the curative factor for a short period of time, significant changes in the urinary

excretion of phosphorus were noted as indicated in Tables
II, III, and IV. In general there was a tendency for the
urinary phosphorus to increase when small doses of the
curative factor were given, and for the urinary phosphorus
to decrease when large doses were given.

The curative factor appears to have an effect on the
phosphorus metabolism which produces the change in phos-
phorus excretion of the animals on the diet deficient in the
"anti-stiffness" factor. The change produced by the 1 gamma
dose on the urinary excretion of phosphorus for the animals
which had been on the diet for two months (Table II, sub-
group D) indicates that the factor may act more rapidly in
animals that have not advanced too far in the deficiency.
The action of the factor can only be surmised; the effect
might be on the processes of absorption, hydrolysis, depo-
sition, or excretion of the phosphorus compounds.

CONCLUSIONS

The urinary phosphorus excretion was determined for guinea pigs on a normal diet, guinea pigs on the diet deficient in "anti-stiffness" factor, and for guinea pigs on the deficient diet treated with the curative factor.

The urinary phosphorus excretion in the control guinea pigs was normal.

The guinea pigs on the diet deficient in the "anti-stiffness" factor have a high urinary phosphorus excretion.

The urinary phosphorus excretion in guinea pigs deficient in the "anti-stiffness" factor was changed when the animals were treated with the curative factor. If a large dose of curative factor was given, the urinary phosphorus level decreased. If a small dose of curative factor was given the urinary phosphorus level increased. The results are based on a short time of treatment.

PART II

The object of this study was to determine whether the curative factor would protect guinea pigs against the typical conditions which develop in the animal when deficient in the anti-stiffness factor.

Thirty young animals of nearly the same age and size were placed on the deficient diet developed by Wulzen[25] *; only four of these animals were females. The four females and four of the males were given, in addition to the basal diet, a supplement of 1 gamma of the curative factor per day per animal; at the end of 3 weeks the dosage was increased to 1.5 gamma per day. The supplement was given to the animals for four months. During the entire time weekly records were made of the weight and stiffness.

The animals which received the curative factor remained normal in appearance during the entire time. They never developed the typical stiffness, and had smooth, glossy hair, and bright lustrous eyes. These animals gained weight at a normal rate.

The eight animals fed the deficient diet plus the curative factor were compared quite closely with eight

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* Diet developed by Wulzen

    10.00 gm. milk powder to each 100 cc. of milk
    00.78 mg. (1 ml.) copper sulfate to each 100 cc. milk
    4.82 mg. (1 ml.) ferric chloride to each 100 cc. milk
  150 I. U. vitamin A in carotene
    6.00 ml. orange juice per 100 gm. weight of animal
  Straw and iodized salt added ad. lib.

animals on the deficient diet without the supplement. Each
animal in the latter group not only showed the wrist stiff-
ness, but had unkempt hair and somewhat dull eyes. Four
animals from each group were killed for autopsy.

The autopsies were made in pairs comparing each animal
that had received the supplement with ones of the animals
from the group which had received no supplement.

Autopsy Records

Animal 1233 compared with animal 1184. Number 1233,
deficient diet plus Curative Factor. Entire body was very
normal, muscle fibers normal microscopically*. Number 1184
much paler muscles than 1233, but no visible calcification
seen in the muscles. Psoas musculature and bracialis
muscles were very white, under microscope no normal stri-
ations seen. All muscles of 1184 were paler in color than
those of 1233 and also had a "dry" appearance when cut.

Animal 1238 compared with animal 1213. Number 1238 on
deficient diet plus supplement; found no deposits of calcium
anywhere and the body was normal. Muscles appeared perfect.
In contrast, 1213, deficient diet only, showed very white
muscles, but no deposits or even streaking of calcium could
be found. Before death 1213 had such degenerated muscles
that he dragged his hind legs. Examined fresh unstained

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* Microscopic study of muscle fibers: Preparations made by
    placing a fragment of muscle in Mammalian Ringer
    solution and teasing out the fibers.

preparations from <u>psoas</u> musculature from both animals;
<u>psoas</u> muscles of 1213 had very few muscle cells which showed
normal striations; cells showed complete disorganization of
structure; muscle fibers of 1238 appeared perfectly normal.

<u>Animal 1237 compared with animal 1186</u>. Animal 1237,
deficient diet plus curative factor. The body of 1237 was
very normal, having red, full muscles with no calcifications
anywhere. Animal 1186, deficient diet with no curative fac-
tor, was diffusely calcified throughout; little strings of
calcium beads running longitudinally with relation to the
muscles, especially seen in all thin muscles of the body
wall and in the <u>serratus</u>. Large calcium deposits were in
the stomach wall, colon and aorta. Microscopic examination
of muscles showed that fibers in <u>psoas major</u> and <u>serratus
anterior</u> of 1186 were degenerated; 1237 showed very normal
muscle fibers; musculature was also darker in color.

<u>Animal 1244 compared with animal 1181</u>. Number 1244,
deficient diet plus curative factor, appeared normal with
no signs of calcification anywhere. Animal 1181, deficient
diet with no supplement; muscles pale and wasted. Saw
spots of calcium in small intestines. Testes, <u>epidiymus</u>
and <u>vas deferens</u> calcified, also <u>seminal vesicles</u>; all
other internal organs were paler in color.

<u>Discussion</u>. It can be readily seen from the above
records that all the animals on the deficient diet showed
some type of calcification; there were differences in

severity and location, but calcification was present. There was no sign of calcification in the group on the deficient diet plus the curative factor.

In order to discover whether any pronounced differences existed between the skeletons of the two groups of animals, the bones were cleared of extraneous tissue and dried.

With the aid of calipers measurements were made on the length of the limb bones and skull and width of femur. The results of the measurements are summarized in Tables I and II.

TABLE I

Length of Limb Bones

Deficient Diet		Deficient Diet ⨎ Curative Factor	
Animal 1186	wt. 686 gm.	Animal 1238	wt. 688 gm.
humerus	3.81 cm.		3.39 cm.
ulna	4.08		lost
radius	3.23		3.22
femur	4.57		4.43
Animal 1186	wt. 686 gm.	Animal 1233	wt. 671 gm.
humerus	3.81 cm.		3.78 cm.
ulna	4.08		3.65
radius	3.23		2.92
femur	4.57		4.39
Animal 1213	wt. 646 gm.	Animal 1233	wt. 671 gm.
humerus	3.77 cm.		3.78 cm.
ulna	4.08		3.65
radius	3.21		2.99
femur	4.54		4.39
Animal 1184	wt. 487 gm.	Animal 1244	wt. 610 gm.
humerus	3.47 cm.		3.62 cm.
ulna	3.67		3.36
radius	2.94		2.97
femur	4.19		4.31
Animal 1181	wt. 775 gm.	Animal 1238	wt. 688 gm.
humerus	3.83 cm.		3.39 cm.
ulna	4.17		lost
radius	3.37		3.22
femur	4.54		4.03

TABLE II

Length of Skull and Width of Femur

Average Weight	Diet	Average Skull Length	Average Width of Femur
648	Deficient plus cure	6.329	0.61
648	Deficient	6.229	0.57
Difference		0.100	0.04

Discussion. The length of the limb bones was found to be greater for animals on the deficient diet with no cur a-tive factor.

The length of the skull and width of the femur appeared to be about the same for the animals from each dietary group.

Summary. Very small doses of the curative factor protected the animals from the usual musculature stiffness and abnormal deposits of calcium. The tissues of the animals from each group were distinctly different. Since bones had not been extensively studied before and the number of animals used was limited, it would seem advisable to carry on further investigation.

CONCLUSION

Animals on the diet deficient in the "anti-stiffness" factor can be protected from the usual symptoms by giving small doses of the curative factor.

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