ALKALINE PHOSPHOMONOESTERASE LEVELS IN THE BLOOD OF GUINEA PIGS (Cavia cobaya) ON A DIET DEFICIENT IN AN ANTI-STIFFNESS FACTOR

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INTRODUCTION

It has been known for over one hundred years that enzymes, organic catalysts, exist in living cells. The chemical mechanism of metabolic transformation in both plants and animals depends upon these substances.

In 1907 Suzuki, Yoshimura and Takaishi (8) reported the existence of an enzyme in rice and wheat bran which was capable of decomposing phytin (the calcium magnesium salt of inositol hexaphosphoric ester) with the liberation of phosphate ions. The following year, 1908, McCollum and Hart (19) demonstrated that animal tissues, such as blood and liver, also contained an enzyme which would hydrolyze phytin. These investigators were not able, however, to demonstrate the presence of this enzyme in muscle or kidney. In 1911 Levene and Medigreceanu (18) tried to demonstrate the presence of phosphatase in extract of intestinal mucosa and kidney, using as a substrate nucleotides. Under the conditions of the experiment it was proved that kidney and intestinal mucosa were very high in this enzyme. Grosser and Hulser (10) found a year later that an enzyme which would hydrolyze glycerophosphate was widely distributed in animal organs. They further noted that kidney and intestinal mucosa contained the highest amount of enzyme of the organs tested. Both the glycerophosphate

obtained by hydrolysis of lecithin and that obtained by synthesis were attacked equally well. In this same year von Euler (41) proved that the mammalian intestinal mucosa and kidney would hydrolyze glucose 1-6 diphosphate and also that, if this substance were fed to dogs, it would be completely hydrolyzed, indicating the same phosphatase activity in vivo.

In 1913 Plimmer (16) proposed that the various phosphatases be divided into groups depending on the number of phosphate ions split off the organic molecule by the enzyme. He demonstrated the existence of an enzyme which would only hydrolyze monophosphates, the phosphomonoesterase. This was followed by Tomita's (39) work in which he showed that tissues from both warm and cold blooded animals would hydrolyze Neuberg's hexose monophosphate.

Robison (23) in 1923 correlated phosphatase activity with ossification of bone. In his classical experiment he noted that phosphatase extract mixed with the easily soluble calcium-barium salt of glucose monophosphoric acid formed insoluble precipitates, $Ca_3(PO_4)_2$ and $Ba_3(PO_4)_2$. The formation of $Ca_3(PO_4)_2$ suggested to him the query of whether, in physiological intact tissue, bone might not be laid down in some such manner. Enzymatic hydrolysis would increase the phosphate ion concentration to a point where the $[Ca^{++}] \times [PO_4^{\equiv}]$ ion concentration would exceed the solubility product, causing precipitation of bone salt.

In attempting to prove his new theory Robison found that the hydrolytic (phosphatasic) power of ossifying cartilage was over ten times as great as that of non-ossifying cartilage. The cortex of the kidney also yielded a very active extract, though less active than forming bone. These striking discoveries pointed directly to the fact that high phosphatase activity in bone-forming matrix, especially in young, growing animals, is very likely an integral part of the ossification mechanism of all bones.

The phosphomonoesterase is the most abundant of the phosphatases in the mammalian body and apparently the most important. In 1931 Roche (27) concluded that this enzyme would hydrolyze almost any monophosphate ester that did not poison the enzyme. He at this time also discovered that di-sodium phenylphosphate was hydrolyzed almost twice as fast as any other phosphate ester tried. Because of its fast hydrolysis Armstrong and King (3) devised a special determination using disodium phenylphosphate as a substrate. It has the advantage over B-glycerophosphate in being quicker, since the reaction time may be shortened. By shortening this period of hydrolysis, the initial reaction rate may be more closely approximated. A modification of the method will be described later.

We must realize that, like all other living constituents of the animal body, bone is in dynamic state. Schoenheimer, in his classical work on the dynamic state of body

constituents, showed that the fat or the amino acid molecule was in a constant state of flux, being continuously degradated and resynthesized. In bone we have histological indications that the same is true. The calcium salts are deposited in the periphery of an ossification center, yet in the center of such an area it is obvious that some of these calcium salts have been removed. To explain this it is convenient to represent the tissue components as part of an equation according to the laws of mass action:

$$\text{Ester} + \text{Water} \rightleftharpoons \text{Alcohol} + \text{Water}$$

The direction of this equilibrium depends upon many factors which have not yet been answered satisfactorily, but the basis for the equation remains the same. Speed of reaction with the formation or liberation of acid, in this case phosphoric acid, will depend upon the enzyme phosphatase.

In 1927 Martland and Robison (20) added weight to this theory by noting that as much as twenty-five per cent of the inorganic phosphate disappeared when the phosphate ion was added to a phosphatase-glycol mixture. Kay (15) in 1928 completed verification of this reversible reaction by isolating glycerophosphate from the above reaction mixture.

Another factor of interest is the possibility of a co-enzyme grouping within the phosphatase molecule. Erdman (6) noted that Mg^{++} ions strongly activate the enzyme in very small quantities. This increase in some cases may a-

mount to over 3000 per cent. Other ions activate or inactivate to a lesser extent.

The optimum pH for phosphomonoesterase has been found (20) to increase with increasing alkalinity; however, the enzyme is rapidly destroyed at higher hydroxyl ion concentrations. The optimum pH, therefore, depends upon the substrate and the duration of hydrolysis; it varies between 8.4 and 9.4.

PHYSIOLOGY

With the previously mentioned facts in mind, we may now concern ourselves with the question of the role of a phosphatase in osteogenesis. Robison's original hypothesis (1923) of osteoblastic secretion of phosphatase has already been pointed out. Another discovery made by Robison and Sloams (26) was that rachitic animals have unusually high phosphatase values. This was interpreted by Robison to mean that in vitamin D deficiency a lack of soluble phosphate esters in the blood supply of the bone was probably the cause of the disease. This they demonstrated to be true by placing such hypertrophied cartilage in a solution containing sufficient calcium and phosphate esters. In such a solution immediate and typical calcification resulted.

Another method of approach in demonstrating the role of phosphatase in ossification was made by Martland and Robison using the human platella. This cartilage anlage does not ossify until the third or fourth year of life. The appearance of phosphatase in chemically detectable amounts coincides remarkably with the first histological signs of ossification. Huggins' (13) experiment is also of interest at this point. In the normal canine bladder epithelium there is a definite but small amount of phosphatase activity. If, however, this epithelium is transplanted into the abdominal muscles (M. rectus abdominalis), ossification of this area will begin. The successful transplants show a

rise in phosphatase coinciding with the beginning of ossification, which is generally about the 18th day.

If Rd^{++} or Pb^{++} ions are accidentally or otherwise introduced into the mammalian body, they will eventually precipitate in the bone as $Me_3(PO_4)_2$. Because the concentration of such metals is generally quite low, it has been assumed that the phosphatase enzyme, by raising phosphate ion concentration, has caused these metal ions to be selectively precipitated in bone.

Fell and Robison (7) have shown by <u>in vitro</u> experiments that there are limitations to the phosphatase ossification mechanism. A definite minimum concentration of phosphate and calcium ions must be present; also a high phosphate ester concentration tends to produce abnormal results.

Despite all these facts which indicate a very close correlation between phosphatase and ossification, there are other disturbing problems which are not yet understood. The cortex of the kidney and the intestinal mucosa have very high phosphatase activity, yet these organs do not ossify normally. The aorta, which has a relatively low phosphatase content, will in some cases be sclerotic. To explain such discrepancy Robison proposed his "second mechanism" theory (7,23,26,28,30,34). The nature of this second mechanism is still very vague; however, Robison believes that it is an enzyme. Harris (12) has suggested the role of glycogen

in calcification. This idea has been carried further by Gutman and Gutman (11). They suggested that phosphorylase and glycogenesis may play a vital role in bone formation either by furnishing a substrate (hexose phosphates) or by actually furnishing the free phosphate ions. Other investigators have not infrequently mentioned the internal pH of bone as playing an important part in osteogenesis; still others have suggested that water changes at the interface may in some way cause Ca^{++} and $PO_4^{\equiv}$ ions to be concentrated, thereby precipitating bone. A "second mechanism" theory, therefore, needs to be further studied.

A NEW DEFICIENCY

About 1936 Wulzen and Bahrs (44) discovered a new deficiency disease in guinea pigs. In this disease the animal characteristically shows a general sclerose condition. Calcium deposits appear paralleling the muscle fibers. Aorta, cartilage and intestine are also subject to rough, unnatural deposits. Bone seems to ossify in a spongy mass rather than as a compact, strong, organized structure.

In such a dietary disease, one in which normal osteogenesis is obviously upset, the possibility of abnormal phosphatase activity appeared quite probable. Armstrong and Banting (2) attribute most blood phosphatase activity to that of bone. By removal of such organs as liver, kidney, intestine, etc., they showed that phosphatase values remain remarkably normal. If the phosphatase values were found to be extremely high, as in the case of rickets, it could be concluded that a general excess of phosphatase caused excessive deposition of calcium salts. This would then be a disease similar to rickets, but one in which sufficient bone substrate was present. If the phosphatase were normal, one would be forced to adopt a new theory or expand one of the existing concepts of a second mechanism. If, on the other hand, phosphatase values were found to be lower, it might indicate that phosphatase is regulatory in function. This would seem logical, for if, as in the case of

rickets, the bone substrate is lacking, phosphatase values may increase twenty-fold, presumably in an effort to deposit calcium.

With these ideas in mind, it was decided to determine the phosphatase values in the plasma of normal and deficient guinea pigs.

EXPERIMENTAL METHODS

In this work about ninety-five individual guinea pigs were used. These animals were divided into three groups. The first of the three was intended as a preliminary experimental group. These animals, twenty in all, were divided as equally as possible between sexes. Five animals were used to obtain normal values and fifteen for experimental dietary values. The animals of this group were placed on the experimental diet the seventh week of post fetal life.

From the experience gained from the first groups, a second group was started. There were sixteen normal animals, eight male and eight female; and sixteen diet-deficient animals, again eight male and eight female. Each set of eight animals was placed in a separate pen, and the animals started on their diets at eight weeks post fetal life.

The third group contained older animals selected from experimental and breeding pens used by Dr. Wulzen. They were twenty months of age plus or minus one month. All had accurately known dietary histories. These animals were classed as to their previous diet as follows: (1) those never on a deficient diet; (2) those on a normal diet for four months previous to being tested and on a deficient diet at least one year prior to their normal diet; (3) and those on a deficient diet eighteen months previous to being tested. These animals numbered nine, ten, and twelve, respectively.

Added to the above were a group of twelve animals run at a later date in order to determine more accurately mean phosphatase values for eight week old animals.

Diets used in this work are the same as Dr. Wulzen has used for the last several years. A very satisfactory diet for developing this stiffness disease consists of a water mixture of commercial skim milk powder, straw in liberal amounts, orange juice, carotene, and adequate mineral constituents. Inasmuch as straw was used as bedding for these animals, they ate most freely of it, thus obtaining the normal roughage to which they are accustomed. Animals on such a diet maintain normal viscosity of fecal excretion. Slight weight variations will be described later.

The normal animals used as a comparison had, in addition to the above diet, a small daily supplement of greens such as kale which contains ample amounts of the anti-stiffness factor. Rolled oats were also substituted in part for skim milk powder. The animals of all groups were at no time starved and had generous amounts of food before them both in the afternoon and morning besides the straw in which they were bedded.

Blood for phosphatase analysis was collected by cardiac puncture. This was accomplished after ether anesthetization. Animals were placed in a battery jar which was fitted with a screen platform. A plug of cotton, saturated with ether, was then placed underneath the platform; thus

it was impossible for the animal, through muscular spasms, to touch his eyes or nose to the wet ether. In this way it required from three to five minutes to anesthetize the animal.

Sodium-potassium oxalate was used as an anti-coagulant. An amount of oxalate solution, calculated on a dry basis, necessary to make a .2 per cent solution when mixed with blood, was pipetted into clean test tubes; these were then evaporated to dryness in a drying oven. Blood was withdrawn by cardiac puncture and mixed with the oxalate before coagulation took place. Samples so collected ranged between 2 cc. and 5 cc., depending largely on the size of the animal. In some 320 successful punctures only four animals were lost.

The frequency of blood collection varied according to the group. Group one animals were bled at the rate of one animal per day, rotating the animals in such a way as to require fifteen days to finish the group. The normal animals of this group were bled irregularly, about two samples a week being taken. In the third week of the experiment rate of collection was doubled for both experimental and normal animals. This rate was continued until the eighth week, after which time collections were irregular. The experiment was discontinued after the seventy-eighth day.

Animals of the second group were bled once a week, eight samples being collected each time. These samples

were divided between male and female, also between normal and diet-deficient animals. This group was run for a period of twenty weeks before the experiment was discontinued.

Blood samples from the third group of animals were not collected in any definite order. Samples were collected at such times as were convenient; this extended over a period of about two months.

Blood samples collected as described were freed of cellular material by centrifugation at 2000 R.P.M. for ten minutes. The plasma so obtained was stored in the refrigerator until used for analysis. In most cases blood was collected between 8:00 A.M. and 10:00 A.M., and phosphatase values were determined between 7:00 P.M. and midnight.

Duplicate phosphatase values on the same sample of blood seldom differed one-tenth of a phosphatase unit. Duplication of results depended upon accurately timed reactions as well as ordinary, careful, analytical technique. Important in timed reactions is the running of a small enough number of samples at one time to avoid confusion. Larger phosphatase differences were found when blood was collected from the same animal at closely spaced intervals.

The method of evaluating the alkaline phosphomonoesterases was essentially that given by Wiese, Johnson, Elvehjem, Hart and Halpin (43), modified to suit the Klett-Summerson photoelectric colorimeter. Reagents were as follows:

A. A buffer substrate composed of 10.3 gms. of sodi-

um veronal and 1.09 gms. of disodium phenyl phosphate made up to one liter. This was preserved in a well-stoppered bottle by a few drops of chloroform and stored in the refrigerator when not in use.

B. Phenol reagent of Folin and Ciocalteu--diluted 1:3.

C. A 20 per cent solution of sodium carbonate.

D. A standard phenol solution containing exactly .1 mg. of phenol per cc.

E. A standard solution and reagent prepared by mixing 5 cc. of phenol (D) with 15 cc. of diluted phenol reagent (B) and by diluting this to 50 cc. with water. This solution contained .01 mg. of phenol per cc.

Analysis was as follows:

Ten cc. of buffered substrate were placed in a water bath at 37.5° C. for five minutes. At the end of that period exactly .5 cc. of blood plasma was added; the contents of the tube were mixed and allowed to stand for exactly thirty minutes in the water bath at 37.5° C. Next, 4.50 cc. of diluted Folin and Ciocalteu's reagent were added and mixed. This mixture was allowed to stand for ten minutes, then was filtered through hard filter paper. Five cc. of the filtrate were then pipetted into a 25 cc. volumetric flask; 2.5 cc. of 20 per cent sodium carbonate solution were then added and made up to volume. Next the mixture

was allowed to stand for thirty minutes while the color developed. At the end of this time a portion was transferred to a colorimeter tube, and the color developed was read. Simultaneously with the above procedure, a blank was prepared on the same sample of blood, using the same quantities of reagents in every instance as outlined above. This sample, however, was not allowed to hydrolyse for thirty minutes; instead Folin and Ciocalteu's reagent was immediately added, stopping all hydrolysis of the disodium phenyl phosphate by precipitation of the protein material in the sample. The blank reading represents free phenol in the blood, the substrate, and the available tryptophane and tyrosine residues of the protein material, as well as the free amino acids, tryptophane and tyrosine. The difference in phenol readings between the blank and the thirty-minute hydrolysis reading represented the activity of the phosphatase enzyme in hydrolysing the substrate. A phosphatase unit was defined as that amount of enzyme per 100 cc. of blood plasma which, when allowed to act on an excess of disodium phenyl phosphate at a pH of nine for exactly thirty minutes, would liberate one mg. of phenol. The standard solution (E) was used to calibrate the photocolorimeter.

Phenol concentrations in these determinations were found to be between .02 and .03 mg. per cc. It was found in practice that a two-minute time interval between individual analyses in any group of determinations was most satisfac-

tory. A blue filter, 6600 Å, was used in all colorimetric readings.

RESULTS

The individual phosphatase values for any one week have been averaged as an arithmetical mean. When two or more determinations were made on an individual blood sample, or when determinations were made on two or more individual blood samples from the same animal within one week, the mean value for the individual animal was calculated before averaging the whole group.

Group I (see Graph I):

Week	Normal Animals Phosphatase Units	Deficient Animals Phosphatase Units
0	7.8	7.8
1	9.3	8.3
2	9.5	8.8
3	13.1	9.6
4	12.0	9.0
5	12.4	9.6
6	9.4	7.8
7	8.6	8.2
8	8.9	8.3
9	9.1	6.7

Group II (see Graph II):

Phosphatase values of the second group represent a larger number of animals. From Table II it is possible to understand the variation in phosphatase values according to the age of the animal; values are as follows:

Week	Normal Animals Phosphatase Units	Normal Animals Weight (Grams)	Deficient Animals Phosphatase Units	Deficient Animals Weight (Grams)
0	7.8		7.8	
4	8.7	468	8.0	400
5	10.2	464	8.0	453
6	10.2	453	7.8	537
7	9.0	544	8.1	513
8	8.6	586	7.9	538
9	9.1	588	6.7	568
10	7.5	608	5.2	630
11	7.0	671	5.7	616
12	7.5	695	6.5	622
13	6.5	667	5.7	660
14	6.0	714	5.0	641
16	6.0	720	4.5	685
20	4.0	749	3.2	717
75	5.6		2.4	

Minimum phenol concentrations were observed to be higher in the deficient animal, indicating a higher concentration of tyrosine and tryptophane residues in the blood of these animals. These values show wide variation, and no tabulation of them will be given.

The third group of animals had values as follows: In older animals (twenty months), never on deficient diet, phosphatase values equal 5.6 units (Standard deviation, σ_x = 1.16). Animals which were on a deficient diet for at least a year prior to their normal diet had phosphatase values of 3.06 units (Standard deviation, σ_x = .743). Those which had been on the deficient diet at least eighteen months prior to being tested had average phosphatase values of 2.4 units (Standard deviation, σ_x = .744).

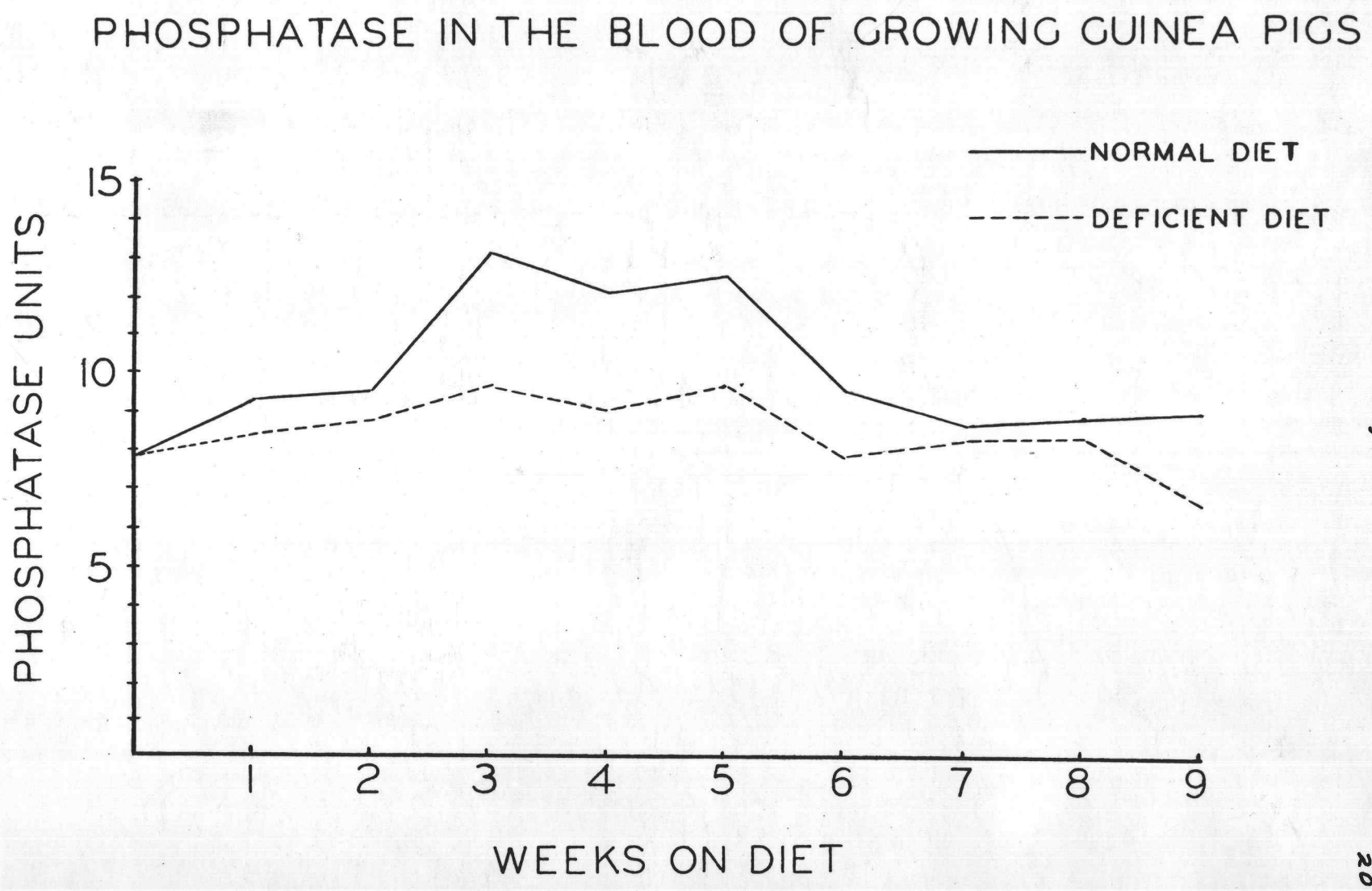

PHOSPHATASE IN THE BLOOD OF GROWING GUINEA PIGS
NORMAL DIET
DEFICIENT DIET
PHOSPHATASE UNITS
15
10
5
1
2
3
4
5
6
7
8
9
WEEKS ON DIET

Fig. 1

PHOSPHATASE IN THE BLOOD OF GROWING GUINEA PIGS

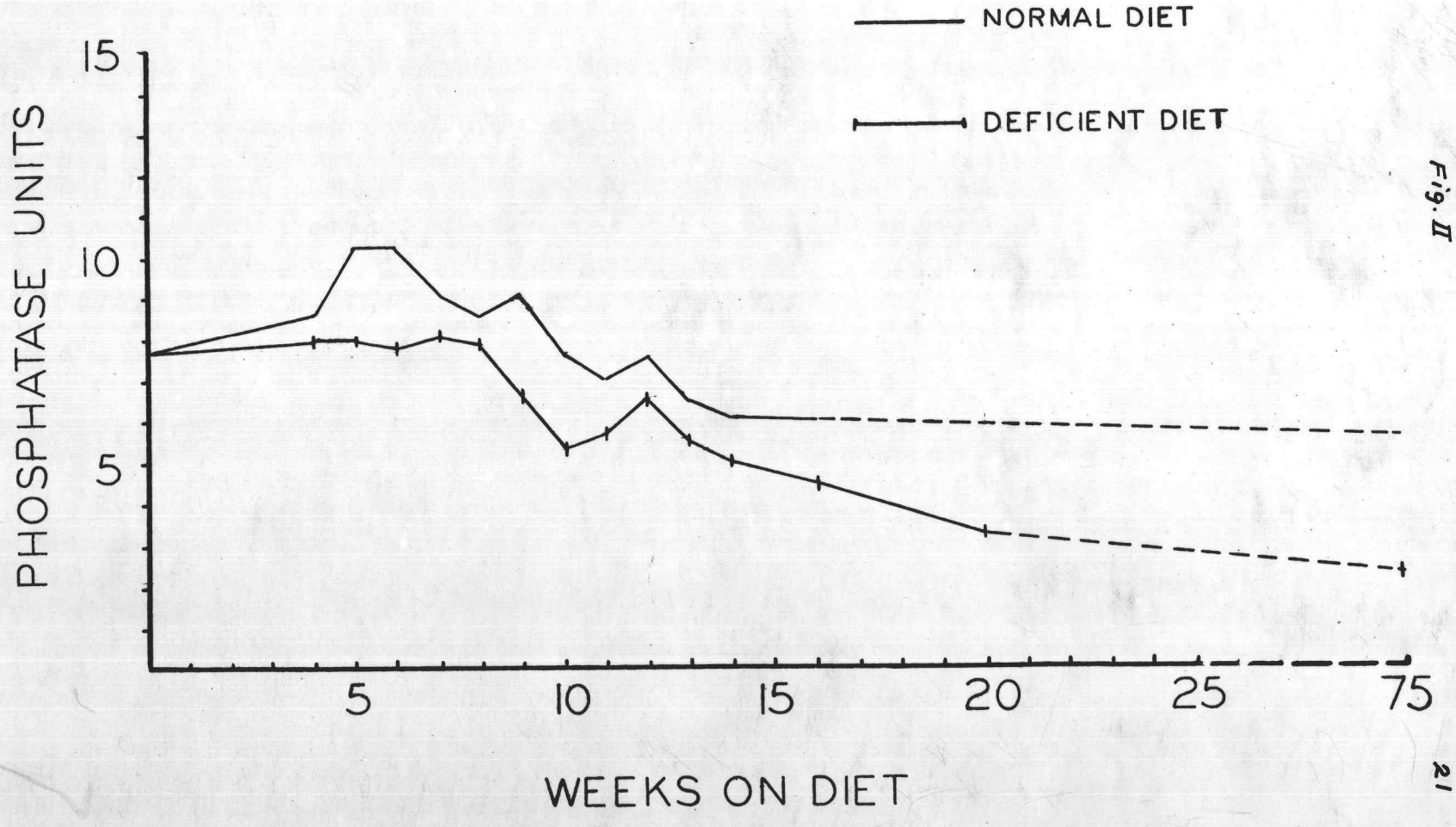
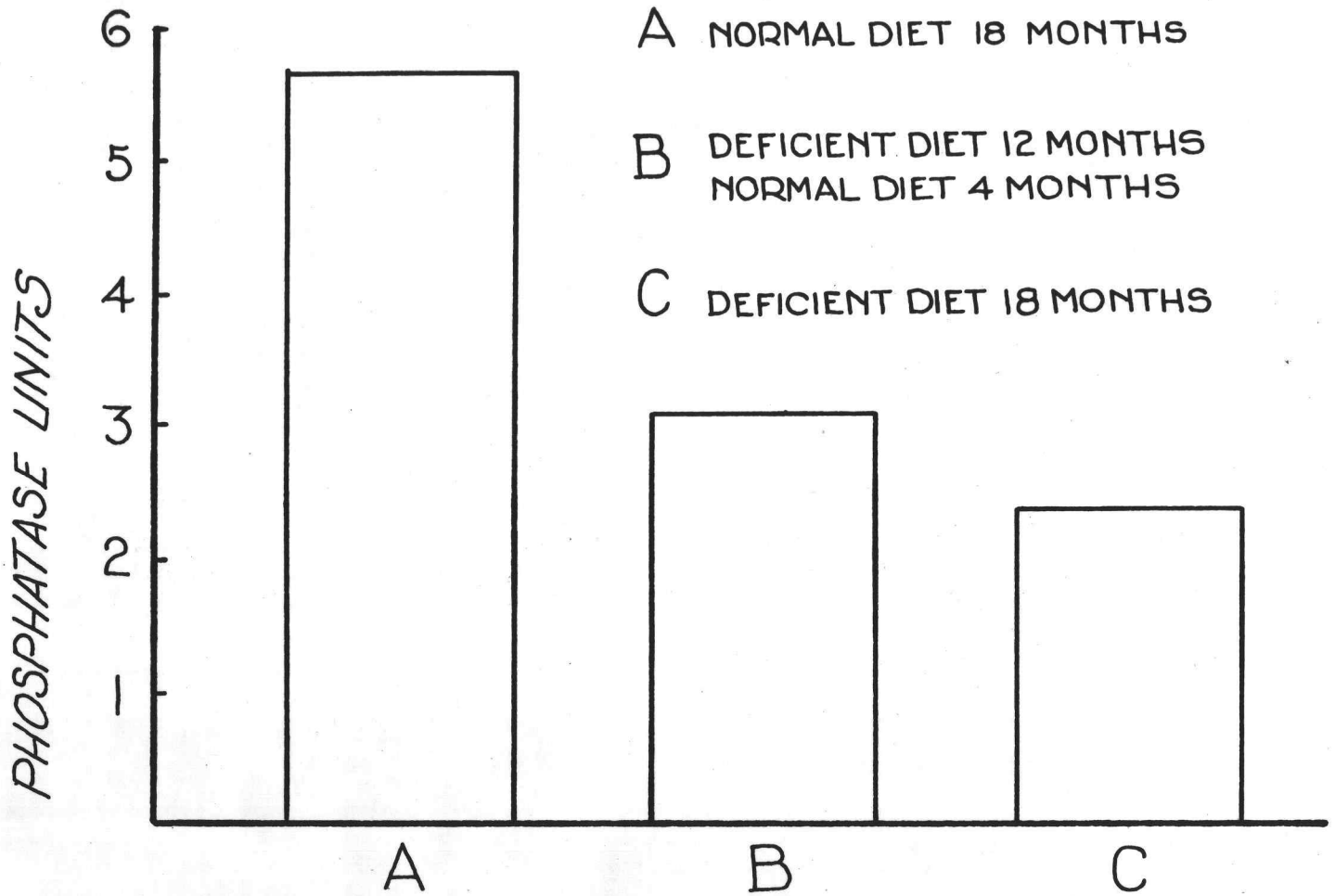


Fig. II



PHOSPHATASE IN THE BLOOD OF GROWN GUINEA PIGS

Fig. III

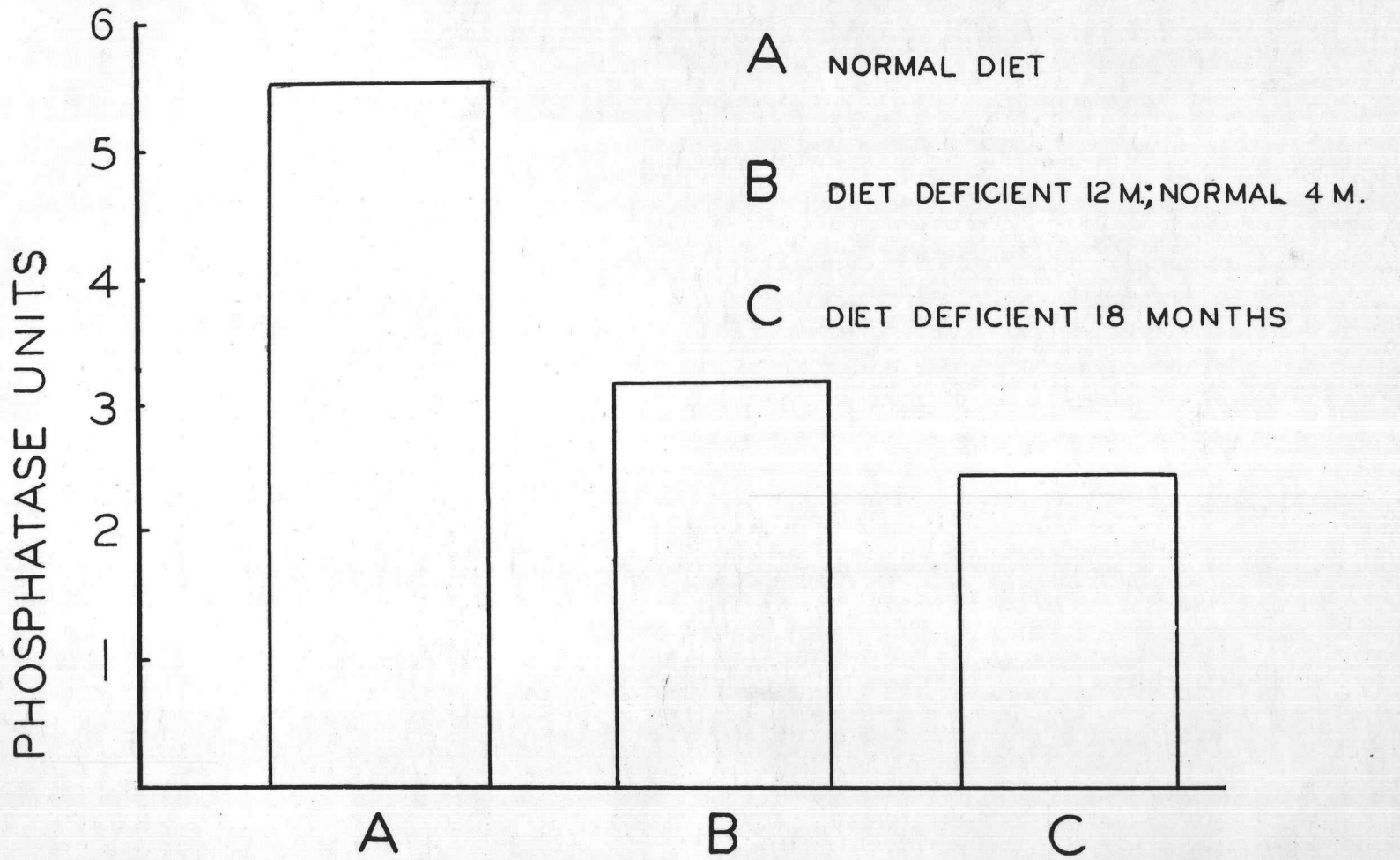


Fig. III

PHOSPHATASE IN THE BLOOD OF GROWN GUINEA PIGS

DISCUSSION

It has been recognized by many investigators that phosphatase values change with age. For this reason it is necessary to compare the experimental animal with the normal animal within definite age limits. In this work the week has been taken as a convenient unit of time in measuring the development of the deficiency symptoms.

By referring to Graphs I and II, it will be noted that animals of group one reach a maximum phosphatase level at an earlier age than those of group two; further, this level is higher in group one than in group two. The explanation of this apparent difference is difficult. Animals of group one were bled more frequently than those of the second group. This definitely has something to do with the development of the condition, for the animals of the first group were less stiff than those of the second group.

Despite slight variations, certain ommon features appear in both groups. Phosphatase values are definitely lower in animals developing this deficiency disease. A definite maximum is reached in phosphatase values in the tenth week of post fetal life in group one, or, in the case of group two, in the thirteenth week. It may be safely assumed that in any large group of guinea pigs this maximum would fall between the tenth and the fourteenth week of life.

After this maximum has been reached, there is a steady

decrease in phosphatase values until the twenty-eighth week of life, after which phosphatase values decrease very slowly.

At the time blood was collected, wrist stiffness (44) was measured. It has been found impossible to correlate this stiffness with phosphatase values. This is unfortunate inasmuch as this has been one of the main methods of measuring the severity of the disease and evaluating the effectiveness of the new vitamin.

Unlike most other blood constituents, plasma phosphatase is not subject to narrow physiological ranges. Armstrong, King, and Harris (4) have experimentally produced obstructive jaundice in dogs and have observed a thirty to one hundredfold rise in alkaline phosphomonoesterase values. The same trend has been noted by Schiffman and Winkelman (31) by ligation of a single hepatic duct.

A most important correlation has been drawn between the various bone diseases and alkaline phosphomonoesterase values as found by blood analysis. Rickets (infantile, adolescent, and renal), osteomalacia, generalized osteitisfibrosa, and osteitis deformas greatly increase phosphatase values proportional to the severity of the disease (5,14,24, 25). Further, it has been found by Stearns and Warweg (36) that plasma phosphatase levels are still high in some cases as long as one year after a child has apparently recovered from rickets. These observations are of great interest be-

cause Dr. Wulzen (44) has repeatedly noticed that the symptoms of this deficiency are aggravated by cod liver oil.

It appears that this anti-stiffness factor may, in many respects, be opposed to vitamin D. In rickets, calcium salts (especially in the young individual) are removed from the tissues; in Wulzen's disease, they are deposited. Phosphatase values, while greatly increased in rickets, are lowered, though less spectacularly, by the deficiency of this dietary factor. As illustrated in Graph III, it has been found that phosphatase values remain low at least four months after an animal has been placed on a normal diet. Contrasted to this is Stearns and Warweg's observation that phosphatase values remain high long after a patient has been cured of rickets.

Despite ample precaution taken to provide adequate amounts of ascorbic acid, it has been suggested by some that the deficiency symptoms are the same as for scurvy. In both diseases long bones show an irregularity of calcium deposition with weakness of trabeculae near the epiphyseal region of the shaft. Todhunter and Brewer (38) have investigated the changes occurring in plasma phosphatase in scurvy. Phosphatase values may rise slightly at the beginning but later drop as much as sixty-six per cent of the normal value. This change has been substantiated by others (32,33). Such changes seem to be the most pronounced in young individuals. Although vitamin C and the anti-stiffness vitamin are both

very susceptible to oxidation, van Wagtendonk (40) has shown them to be different substances by isolating a fat-soluble compound, in contrast to water soluble ascorbic acid. Ease of oxidation of the two vitamins, low phosphatase values and weak trabeculae then appear to similarize the two vitamins. Water solubility of ascorbic acid, with extravasation of blood and the production of a severe edema in scurvy differentiate the two substances. Other differences between the two vitamins might be pointed out.

The development of stiff tendons and general joint stiffness is obviously, by definition, an arthrosis, more loosely named, a dietary arthritis. It is of interest to compare phosphatase values as found in various clinics. At the Arthritic Clinic, Rochester General Hospital, Steinberg and Suter (37) have determined phosphatase values for several types of arthritis. Normal individuals have three Bodansky units of alkaline phosphatase, hypertrophic arthritic patients 2.6 units, and atrophic arthritic patients 1.8 units. These values compare favorably with our results, indicating a possible similarity of pathology. Others (1,35) have found phosphatase levels in arthritis to vary above and below the normal value and conclude that phosphatase results are insignificant. Watson (41), however, has reported an actual rise in phosphatase levels resulting from non-specific arthritis. It may be said that clinical material so far has been largely restricted to elderly people. This tends to

include many individuals with cancer of the prostate and other general cancerous lesions of subclinical nature. These diseases tend to raise serum phosphatase averages of clinical cases.

Other vitamins that may be associated with a similarity of pathology have little effect on phosphatase levels.

Experimental results, as shown in Graphs I and II, are of little value in analysis of this disease or its progress. In older animals, Graph III, phosphatase values may be used to distinguish between the normal and the experimental animal; however, the wrist test is so much simpler, and apparently more satisfactory, that the long and laborious phosphatase determination would be useless. Inasmuch as the standard deviation, $\sigma_{\bar{x}}$, is about one unit, it is obvious that only an average of several individuals has any significance.

Of questionable importance is the irregular increase of phenol derivatives in the blood of the diet-deficient animal. The possibility that, in this deficiency, the concentration of amino acids in the blood is increased should not be overlooked. It will be noted, however, that analysis by this method measures only tryptophane and tyrosine. It would not imply, therefore, an increased concentration of all amino acids of the blood stream. It will be seen that there small weight differences between dietary and normal animals. It is believed that these differences are small enough to be

of little or no significance.

SUMMARY

1. Alkaline phosphomonoesterase activity in the plasma of guinea pigs has been followed in normal animals and in animals developing a dietary stiffness disease.
2. It has been found that in normal animals phosphatase values reach a maximum in the tenth to the thirteenth week of post fetal life.
3. In both normal and experimental animals, phosphatase values decline steadily from their maximum until the twenty-eighth week of life, after which time there is but slight lowering in phosphatase activity.
4. Plasma phosphatase levels are lower in experimental animals on a stiffness-producing diet. After eighteen months phosphatase values are about fifty per cent lower in the deficient animals.
5. Phosphatase values do not immediately return to normal after the animal is placed on a normal diet; even after a period of four months they will be found significantly lower than normal.
6. It appears that this dietary factor is similar in its effect to vitamin C and opposite in its effect to vitamin D in respect to influence of phosphatase levels and some aspects of general pathology.
7. Phosphatase activity appears to vary inversely with calcium salt deposition in bones. It may possibly play a

regulatory role in ossification, depending upon the activity of other "second mechanism" factors.

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