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An In Vitro Study of the Effects of Parathyroid Hormone on the Activity of Alkaline Phosphatase

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AN IN VITRO STUDY OF THE EFFECTS OF

PARATHYROID HORMONE ON THE ACTIVITY

OF ALKALINE PHOSPHATASE

by

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LIFE

Michael Joseph McCormack was born in Chicago, Illinois, November 2, 1937.

He was graduated from St. George High School Evanston, Illinois, June, 1955. After attending the Illinois Institute of Technology, Amundson Junior College, Wright Junior College, and DePaul University, the author graduated from the University of Illinois with the degree of Doctor of Dental Surgery in June, 1966.

He entered the Department of Orthodontic of Loyola University in June, 1966 and began his graduate studies in the Department of Oral Biology in September, 1966.

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CHAPTER I

1. REVIEW OF THE LITERATURE

A. ALKALINE PHOSPHATASE

The history of alkaline phosphatase research began with the work of Robison (47) in 1923, who described an enzyme which was present in the ossifying cartilage of young rats and rabbits which rapidly hydrolysed hexosemonophosphatic acid and yielded free phosphoric acid. Further proof of its connection with calcification was the fact that nonossifying cartilage showed less than one tenth of the hydrolytic power of ossifying cartilage. On the basis of these observations, he theorized that the liberation of a phosphate ion from an ester would cause the percipitation of calcium phosphate by increasing the concentration of the phosphate ion in that area of bone. Robison and Soames (48) found this enzyme to be present in bone, teeth, intestine and hypertrophic cartilage. They also found the optimum activity of this enzyme to be between pH 8.4 and 9.4. Further support for the role of this enzyme in ossification came from thei experiments with the bones of rachitic rats (48). These were placed in a

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solution of calcium hexosemonophosphate or glycerophosphate at $37^{\circ}C$, pH of 8.4 - 9.4, and after a period of time, areas of deposition of fresh calcium phosphate were observed. They concluded that the enzyme was possibly secreted in the osteoblastic and hypertrophic cartilage cell regions, which accounted for this fresh deposition.

Diagnostic studies with the blood levels of this enzyme in disease states, particularly in bone disease, were conducted by Kay (27), who found that the three most active sites of alkaline phosphatase were intestinal mucosa, kidney, and the epiphysis and periosteum of bone. In certain bone diseases such as osteitis deformans, generalized osteitis fibrosa, osteomalacia, and rickets, the activity levels of alkaline phosphatase was shown to be 20 times the normal level. He concluded that the high plasma phosphatase levels were the result of bone lesions rather than its cause since the majority of tissues contained alkaline phosphatase as an intracellular enzyme which probably functioned in maintaining the equilibrium between the inorganic phosphate levels and the constitutional phosphoric esters of the cell. This later group include the nucleic acids, RNA, DNA, and the simpler acid-soluble phosphoric esters (27). He also devised a method for the determination of phosphatase activity in blood (26).

In 1941 Gutman and Gutman (20) did a study on phosphorylase in calcifying cartilage, and concluded that there appeared to be a phosphorylative, glycogenolytic enzyme system capable of snythesizing potential substrates for bone phosphatase at zones of calcification. This was observed in the area of hypertrophied epiphyseal cartilage. The first study of serum phosphatase activity in hyperparathyroidism was done by Klendshoj and Koepf (29) in which they found that both alkaline and acid phosphatase levels were higher in patients with adenomas of the parathyroid gland, and these levels could be reduced to normal following extirpation of the gland. They finally concluded that bone most likely supplied the major part of serum alkaline phosphatase since the serum level was not reduced when other organs were surgically removed. Gomori (18) found that zones of calcification in tissues appeared most active in those areas intensely alkaline phosphatase positive.

Working with slices of epiphyseal cartilage from long bones in young rachitic rats, Waldman (57) was able to demonstrate calcification in vitro following the inactivation of phosphatase and other enzymes. He concluded that calcification was intrinsically non-enzymatic and that the phosphorylase and phosphatase systems discussed by Gutman and Gutman (20) had supplementary roles in calcification and were not essential to

localized deposition of bone mineral. Siffert (50) reached the same conclusion, that the enzymes appeared to be intimately related to pre-osseous cellular metabolism and to the elaboration of a bone matrix that was chemically calcifiable but calcification was able to occur with or without the presence of these enzymes. He further concluded that phosphatase was physiologically active only in the presence of living cells.

Jones and Shinowara (25) observed significant difference between the alkaline phosphatase activity of hypophysectomized rats and non-hypophysectomized rats. The activity was relatively higher in the hypophysectomized group, however, the rise seen in the control group was attributed to growth which was in agreement with similar observations of Weil (58).

B. PARATHYROID HORMONE

The parathyroid glands were first described by Sandstrom (49) in 1880. He discovered the external glands but it was not until 1895 that the internal parathyroids were recognized by Kohn (31). Prior to this time thyroidectomies were performed without the realization that more than one organ was being removed and the tetany which followed each operation was attributed to a loss in function of the thyroid gland. In 1908,

MacCallum and Voegtlin (34) found that the tetany associated with the removal of the parathyroid glands were controlable through the administration exogenous calcium, and thus established a relationship between the nervous manifestation of parathyroidectomy and the serum calcium levels.

In the early 1920's much clinical data was collected on hypoparathyroidism, and Mandl (37) in 1925, performed the first parathyroidectomy. Hanson (22, 23) in 1924, and Collip (14) in 1925, independently were able to isolate a crude, but physiologically active principle from the parathyroid glands. Collip (15) obtained his extract from the parathyroid glands of oxen, and was able to demonstrate its affect in controlling tetany in parathyroidectomized dogs. The active ingredient in Collip's extract produced its effect by restoring serum calcium to normal levels, however, he also found that an overdose of this extract produced a state of hypercalcemia. Both the importance of the extract and its effect on serum calcium levels had been established. It seemed now that the important task ahead was to determine the mode of action of this hormone.

C. MODE OF ACTION OF PARATHYROID HORMONE

Albright (1, 2) collaborated with many men in the 1920's and 1930's

to present clinical findings in disease states of hyper- and hypoparathyroidism. His writings thus resulted in his becoming the foremost member of what was known as the "kidney school" of parathyroid action. This group felt that the primary action of parathyroid hormone was on the reabsorption of phosphate by the kidney tubules. Their theory held that as parathyroid hormone levels in the serum increased, the renal threshold for phosphate resorption was elevated, thus the amount of inorganic phosphate reabsorbed by the tubules was decreased, thereby resulting in a phosphate diuresis. This, in turn, caused the Ca x P solubility product of the serum to become altered, permitting more Ca and P to come into solution. They finally felt that the subsequent hypercalcemia observed was secondary to the primary hypophosphatemia.

The other school of thought, relative to the primary function of parathyroid hormone, was the "bone-cell school". This group (16, 40, 43, 52, 56) held that the parathyroid hormone acted directly on bone to cause the release of calcium and phosphate. This resulted in the increased calcium levels of blood serum and explained the increased phosphate in the urine as a secondary effect. The strongest evidence to support this theory was the work of Barnicot (4) in 1948, and Chang (12) in 1951. In their experiments, vital parathyroid tissue was grafted in the

area of the temperal bone of rats and mice, along with other tissues used as controls. The bone in direct opposition to the parathyroid gland showed resorption while layers of new bone were deposited on the other side and in proximity to the other tissues used as controls. Bartter (5) discussed the several conflicting opinions which existed in the literature between 1948 and 1954, and by the time Munson, Hirsch and Tashjian (42) did their comprehensive review of the literature in 1963, most investigators had adopted the "bone-cell theory". The next step seemingly was to determine just how parathyroid hormone affected the release of calcium salts from the bone.

Carnes (11) in 1950 stated that the hormone worked directly on the organic matrix of bone. He observed that bone matrix was destroyed by the action of parathyroid hormone irrespective of its mineral content, and that the levels of serum calcium were a reflections of the mineral content of the resorbed matrix. McLean (38) summarized certain positive factors which led him to the opinion that the hormone worked directly on bone. The fact that the hormone was still effective in totally nephrectomized animals was forceful evidence along with the transplant experiments of Barnicot (4) and Chang (12). Another fact was the demonstration in dogs treated with parathyroid hormone, that serum calcium levels continued to

rise to such a level that the calcium and phosphate concentrations were sufficient to induce calcification in rachitic cartilage in vitro. McLean and Urist (40) introduced the feedback mechanism of the parathyroid glands necessary to maintain the serum calcium levels at 10 milligrams per cent. They were of the opinion that the relatively stable fraction of bone mineral rather than the small exchangeable "labile" fraction was the primary target of the hormone. Talmage and Elliott (52) working with radioactive Ca^{45}, were able to confirm that the hormone worked on the stable fraction of bone mineral.

While the majority of investigators felt that the primary target of parathyroid hormone was bone, other investigators were still studying its effect on kidney. Handler, Cohn, and DeMaria (21) showed that an intravenous injection of the hormone caused inhibition of phosphate resorption and phosphaturia. Beutner and Munson (6), working with rats, found that urinary phosphate levels decreased following parathyroidectomy and increased following injection of parathyroid extract. While some claimed that the increase in phosphaturia was due to an increase in glomerular filtration rate caused by the crude extract, Lavender et al (32) provided evidence for the tubular action of the hormone by infusing 10 to 30 USP units of highly purified parathyroid hormone per hour into one renal artery

of an intact dog, and were able to show unilateral or preferential phosphaturia from the kidney on the infused side. Talmage and his associates (54) were the first to suggest that parathyroid hormone might act on the kidney in such a way as to conserve calcium. Following administration of the hormone to rats, serum calcium levels rose as urinary calcium levels were reduced. Similar findings were reported by Buchanan and his co-investigators (10), and by Kleeman, Widrow and Levinsky (28).

In McLean's (38) "feedback mechanism" theory, the labile fraction of bone mineral was able to maintain the calcium ion concentration in the serum at 7.0 milligrams percent. The action of the parathyroid hormone was necessary for the final 3.0 milligrams percent, to raise the calcium ion level to the normal level of 10 milligrams percent. When the calcium ion concentration was below the normal level for an individual, the gland would be stimulated to secrete more hormone, and when the level was above 10 milligrams percent, the activity of the gland was reduced and less hormone was secreted into the circulation. These postulates agreed with the earlier findings of Patt and Lockhardt (45). Support for the feedback mechanism has more recently come from Copp (16) and others, who concluded that the feedback mechanism alone could not account for the rapid change observed in serum calcium levels in their experiments with

the extract and gland removal. They postulated the existence of a second hormone and named it calcitonin. Copp et al (17) did perfusion experiments to determine the source of calcitonin, and concluded that it was produced by the parathyroid glands and not the thyroid as was originally thought by MacIntyre and his associates (36).

2. STATEMENT OF THE PROBLEM

It seems to be well documented that serum alkaline phosphatase activity increases in certain bone diseases such as osteitis deformans, generalized osteitis fibrosa, osteomalacia, rickets and in pseudohyper-parathyroidism (24, 27, 29). It is likewise well known that two phos-phatase enzymes play an important role in bone homeostasis, acid phos-phatase in bone resorption, and alkaline phosphatase in bone deposition. It must be assumed, however, that normal levels of serum alkaline phos-phatase must be derived from the constant turnover of bone, or other active metabolic sites, such as kidney, which contain this enzyme.

In view of what has already been reported in the literature, relative to increased alkaline phosphatase activity observed in clinical hyperparathyroidism, it was felt that an _in vitro_ study might reveal some additional information heretofore considered trivial if not unimportant.

This in vitro method of study was employed for the reasons that the enzyme-hormone system under investigation would not be effected by such substances as other serum enzyme systems, binding by serum proteins (24), other hormones, and numerous other substances normally found in animal serum. In essence, the primary purpose of this investigation was to determine whether the addition of parathyroid hormone to an isolated alkaline phosphatase-sodium-beta-glycerophosphate system, would have some demonstrable effect on the activity of the enzyme in vitro, and hence account for increased activity observed in experimental, as well as clinical hyperparathyroidism.

CHAPTER II

MATERIALS AND METHODS

This investigation utilized a modification of the original Bodansky method for the determination of alkaline phosphatase activity (7, 8), with sodium-beta-glycerophosphate as substrate. The method of inorganic phosphate determination was essentially that of Sumner (51) where ferrous sulfate was used as the reducing agent.

A. INORGANIC PHOSPHATE DETERMINATION

PREPARATION OF SOLUTIONS

1. Phosphate Standard:

The phosphate standard was prepared by adding 1.0977 grams of previously dried KH_2PO_4 (Baker's, C.P., lot number 51749) in a 250 milliliter volumetric flask which contained 200 milliliters of distilled water. To this was added 10 milliliters of 7.5N sulfuric acid and sufficient distilled water to bring the solution to volume. Each milliliter of this solution contained 1 milligram of inorganic phosphate.

2. Working Phosphate Solution:

The working phosphate was prepared fresh daily by taking 1 milliliter of the phosphate standard solution, previously described, and diluting it to 100 milliliters volumetrically.

3. 7.5N Sulfuric Acid (H_2SO_4):

This acid solution was prepared by slowly adding 104.16 milliliters of concentrated sulfuric acid (H_2SO_4) in 400 milliliters of distilled water. The mixture is allowed to cool and slowly brought to volume in a 500 milliliter volumetric flask.

4. 10% Trichloroacetic Acid:

The solution was prepared by dissolving 100 grams of trichloro-acetic acid in 800 milliliters of distilled water in a volumetric 1 liter flask, mixing, and bringing it to volume.

5. 6.6% Ammonium Molybdate:

The ammonium molybdate was prepared by dissolving 66 grams of the crystals in 500 milliliters of distilled water and taking to volume in a 1 liter volumetric flask.

6. Working Acid-Molybdate Solution:

The working acid-molybdate mixture was prepared fresh daily by adding 1 volume of 6.6% ammonium molybdate to 1 volume of 7.5N sulfuric acid and mixing.

7. 10% Ferrous Sulfate:

The reducing agent was prepared fresh for each set of determination by dissolving 3 grams of ferrous sulfate with 24 milliliters of distilled water. While mixing, 0.6 milliliters of 7.5N sulfuric acid was added and the solution was taken to a volume of 30 milliliters.

SUMNER'S METHOD (51)

From the working phosphate solution previously described, a 1 milliliter aliquot was transferred to a 100 milliliter volumetric flask and diluted to volume with distilled water. From this solution, which contained 0.01 milligram of inorganic phosphate in each milliliter, aliquots from 0.5 milliliter to 5.0 milliliters respectively were placed in 10 labeled cuvettes. To each of these cuvettes the following solutions were added: 1 milliliter of 10% trichloroacetic acid; distilled water in sufficient amounts to raise the volumes of each cuvette to exactly 8 milliliters; 1 milliliter of the acid-molybdate solution, and 1 milliliter of

ferrous sulfate. The contents were gently mixed and allowed to stand for 5 minutes to ensure proper color development. The optical density was read using the Coleman Universal Spectrophotometer at a wavelength setting of 550 millimicrons, (fig. 5, appendix, page 43).

B. ALKALINE PHOSPHATASE DETERMINATION (7, 8)

PREPARATION OF SOLUTIONS

1. Preparation of Enzyme Solution:

The alkaline phosphatase (Mann Research Laboratories, Analytical Lot No. S4624) used in this study was prepared from calf intestine. A solution was prepared fresh for each set of determinations by adding 100 milligrams of alkaline phosphatase powder, which had been carefully weighed, to 80 milliliters of buffered sodium diethylbarbiturate (Merck) solution in a 100 milliliter volumetric flask, mixed and taken to volume.

2. Barbiturate Buffer, pH 8.5

The buffer solution was prepared by dissolving 4.24 grams of sodium diethylbarbiturate (Merck) in 950 milliliters of distilled water in a 1 liter volumetric flask, adjusting the pH to 8.5 with dilute sodium hydroxide and taking the solution to volume.

3. The Substrate Solution:

The substrate was prepared by dissolving 500 milligrams of sodium-beta-glycerophosphate (Mann Research Laboratories, Lot No. S2062) to 80 milliliters of distilled water in a 100 milliliter volumetric flask, mixing well and bring it to volume.

4. Parathyroid Hormone Solution:

The parathyroid hormone powder was obtained from the Mann Research Laboratories (Lot No. T-1717). The fat was removed from this acetone-dried powder after the manner described by Aurbach (3) with a final yield of 70.4 per cent the original weight.

The working solution was prepared by adding 100 milligrams of the fat-free powder to 80 milliliters of distilled water in a 100 milliliter volumetric flask, mixed well, and brought to volume.

C. ASSAY OF THE ALKALINE PHOSPHATASE (7, 44)

From the freshly prepared solution of alkaline phosphatase, a carefully measured amount was placed into each of several test tubes using volumetric pipettes. To each of these was added 1 milliliter of 0.05N magnesium chloride solution. Distilled water was then added and

the mixtures allowed to reach incubation bath temperature ($37^{\circ}C \pm 1^{\circ}C$).
The substrate was then carefully added and the tube was maintained at
$37^{\circ}C \pm 1^{\circ}C$ for exactly one hour in a thermostatically controlled water
bath. At the end of one hour, 1 milliliter of 10% trichloroacetic acid was
added to each tube and the tubes were removed from the water bath and
cooled to stop the further activity of the enzyme. The resulting mixture
was then filtered through a 9 centimeter Whatmann No. 40 filter paper,
after which 1 milliliter aliquots were taken from each collecting tube and
volumetrically transferred into cuvettes, and the inorganic phosphate
determined in the manner previously described. Although the color
developed rapidly, no readings were made on the spectrophotometer until
5 minutes had elapsed following the addition of the ferrous sulfate. The
optical density readings were made utilizing a blank which contained
distilled water, acid-molybdate and ferrous sulfate. Each reading was
made twice and the zero point was checked against the blank before and
after each reading. Simultaneous standards were also run to test the
validity of the determination. The activity which confirmed the Mann
Assay, was found to be 0.22 units per milligram of enzyme, or 650
micromoles of phosphorus liberated.

1. Effect of Variation of Substrate Concentration:

The first studies were done to analyze the effect of various concentrations of substrate on the total inorganic phosphate output with a constant amount of enzyme. Into each of nine test tubes, 5 milliliters of enzyme was carefully measured. To these were added 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 milliliters of substrate respectively. The reaction was allowed to proceed for one hour and the amount of inorganic phosphate liberated was determined as previously described.

2. Effect of Variation of Enzyme Concentration:

In holding the concentration of the substrate constant by the addition of 5 milligrams of sodium-beta-glycerophosphate, the activity of different concentrations of alkaline phosphatase was studies. The enzyme was carefully pipetted into each of six test tubes, varying in amount from 0.5 to 5.0 milliliters with one tube with no enzyme. The reaction was permitted to proceed for one hour and the phosphate determinations done as previously described.

3. Effect of Variation of Magnesium Concentration:

A set of inorganic phosphate determinations was done to study the effect of the coenzyme on the activity of alkaline phosphatase. The concentration of the enzyme was maintained by adding 2 milliliters of the

alkaline phosphatase solution to each test tube along with 2 milliliters of substrate. The concentration of the coenzyme was varied by adding from 0.0 to 2.0 milliliters of 0.05N magnesium chloride solution. The enzyme activity was determined as previously described.

4. Time-Course Study:

The rate of alkaline phosphatase activity was studied by maintaining the concentration of all factors involved except for the amount of time the activity was allowed to proceed. Each of 12 test tubes received the following: 2 milliliters of alkaline phosphatase; 2 milliliters of substrate; 1 milliliter of coenzyme and 5 milliliters of distilled water. All the tubes were placed in the controlled water bath at $37^{\circ}C \pm 1^{\circ}C$. At each 5 minute interval thereafter, 1 tube was removed, the reaction stopped, and the amount of enzyme activity determined as previously described. This was done for each of the 12 tubes to study any variations which might occur in the normal one hour incubation period.

D. THE EFFECT OF PARATHYROID HORMONE
ON ALKALINE PHOSPHATASE ACTIVITY

Into each of 10 test tubes 2 milliliters of enzyme was carefully pipetted. To these tubes, amounts of parathyroid hormone solution were

added varying from 0.2 milliliters to 5.0 milliliters along with 1 milli-

liter of magnesium chloride and sufficient distilled water to raise the

volume in each tube to exactly 8 milliliters. When the solutions reached

incubation temperature, 2 milliliters of substrate were added, solutions

gently mixed and the time noted. After exactly one hour the reaction was

stopped, the solutions filtered and the amount of inorganic phosphate

determined as previously described.

CHAPTER III

RESULTS

A. ALKALINE PHOSPHATASE ACTIVITY

The results of this study proved to be relatively consistent, in spite of the fact that several determinations were made varying only slightly the enzyme-substrate ratios. Consistently the values obtained agreed with those supplied by the Mann Research Laboratory: activity was 0.22 units per milligram, or 650 micromoles per 10 milligrams of enzyme. Due to the consistency of these results, it was felt that the other studies could then be justifiably undertaken.

B. EFFECT OF ENZYME CONCENTRATION

The values which appear in the following graph confirm the fact that increasing the enzyme concentration while maintaining the substrate concentration constant show increased hydrolysis of the latter. A graph of the values shows a linear relationship to exist.

TABLE I

EFFECT OF ENZYME CONCENTRATION

ON HYDROLYTIC ACTIVITY

mg. ALK. PHOS.	$1/E$	AVG. O.D.	mg. INORG. PHOS.	$1/V$
0.5	2.00	0.163	0.156	6.41
1.0	1.00	0.252	0.242	4.13
2.0	0.50	0.330	0.317	3.15
3.0	0.33	0.371	0.355	2.81
4.0	0.25	0.403	0.385	2.59
5.0	0.20	0.419	0.402	2.48

E = Enzyme Concentration   V = Velocity of the Reaction

O.D. = Optical Density

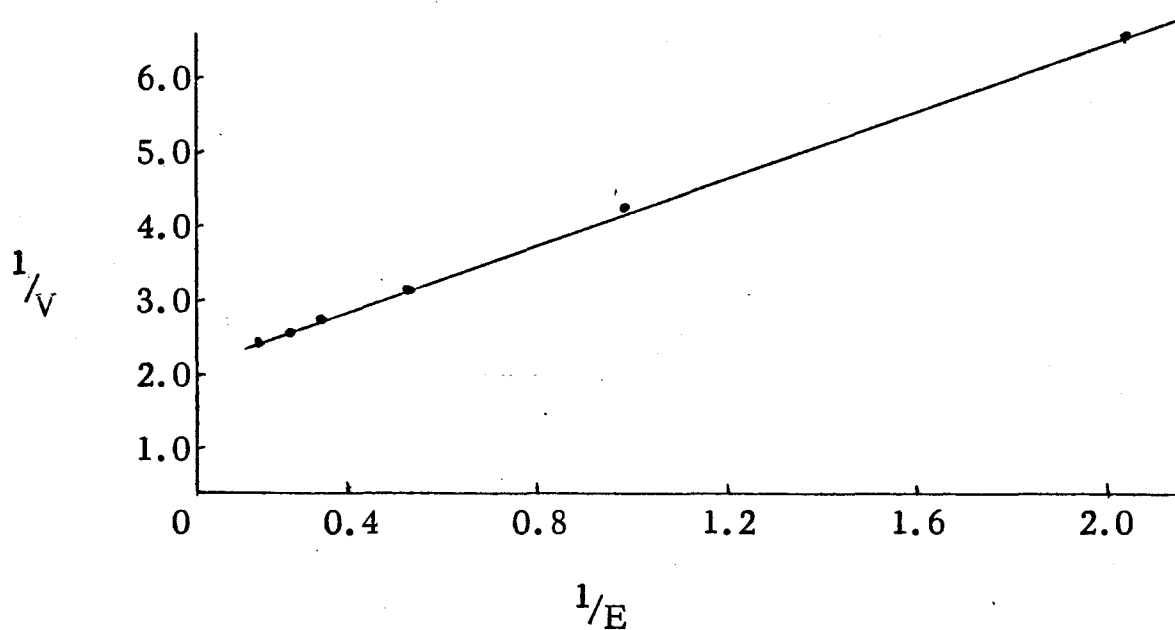FIG. 1. EFFECT OF ENZYME CONCENTRATION

C. EFFECT OF SUBSTRATE CONCENTRATION

A summary of the results appearing in the table below show a consistent rise in the amount of inorganic phosphate liberated as the concentration of the substrate was increased. The 5 milliliters of alkaline phosphatase was sufficient to hydrolyse the greater amounts of substrate present in some of the last tubes. A near linear relationship existed between the amount of phosphate liberated and the amount of substrate present.

TABLE II

EFFECT OF SUBSTRATE CONCENTRATION

ON HYDROLYTIC ACTIVITY

ml.* SUBSTRATE	OPTICAL DENSITIES				AVG. O.D.	mg INORG. PHOS. x 10
	1	2	3	4		
0.5	0.250	0.248	0.248	0.253	0.250	0.240
1.0	0.424	0.420	0.424	0.419	0.422	0.404
2.0	0.689	0.680	0.684	0.690	0.686	0.658
3.0	0.88	0.89	0.87	0.87	0.88	0.845
4.0	1.10	1.11	1.10	1.09	1.10	1.05
5.0	1.28	1.30	1.30	1.32	1.30	1.25

* SUBSTRATE CONCENTRATION: 5 milligrams per milliliter

ENZYME CONCENTRATION: 5 milligrams per tube - constant

A plot of these data shows a tendency for a linear relationship to exist between the amount of substrate and the amount of inorganic phosphate released.
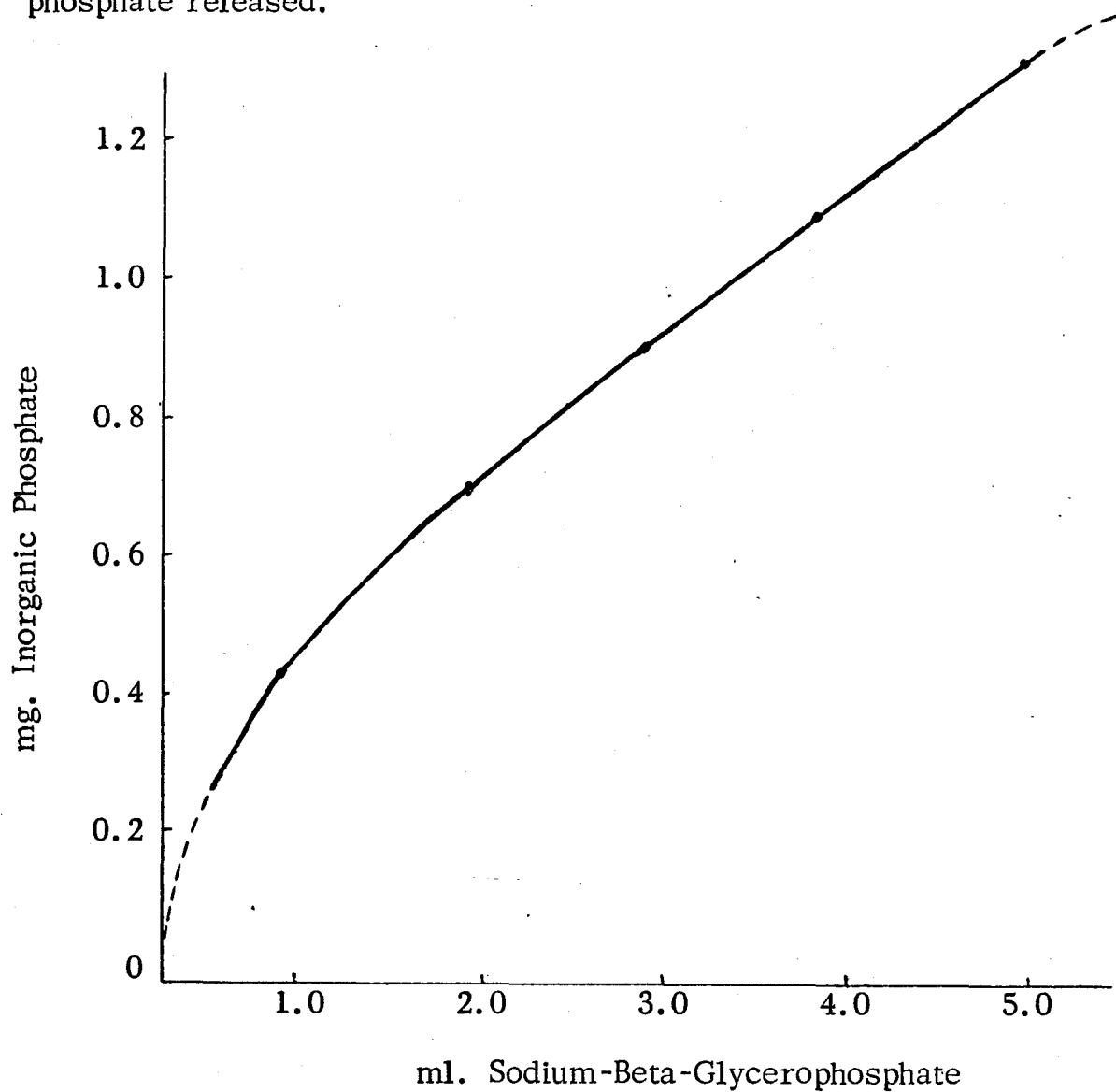

FIG. 2. EFFECT OF SUBSTRATE CONCENTRATION

D. TIME-COURSE STUDY

The results of the study in which time was the variable factor are listed below in table III. An illustration (see fig. 3, page 26) of the results shows an approximately linear relationship between time and the amount of inorganic phosphate liberated by the activity of the alkaline phosphatase.

TABLE III

EFFECT OF TIME ON HYDROLYTIC ACTIVITY

TIME *	OPTICAL DENSITIES			AVG. O.D.	mg. INORG. PHOS. x 10
	1	2	3		
5	0.160	0.154	0.150	0.155	0.150
10	0.202	0.210	0.204	0.205	0.197
15	0.270	0.269	0.268	0.269	0.258
20	0.310	0.304	0.316	0.310	0.297
25	0.347	0.345	0.349	0.347	0.332
30	0.375	0.370	0.379	0.377	0.362
35	0.444	0.435	0.440	0.440	0.421
40	0.497	0.492	0.487	0.492	0.472
45	0.512	0.507	0.502	0.507	0.485
50	0.529	0.527	0.520	0.525	0.504
55	0.552	0.549	0.557	0.553	0.531
60	0.580	0.576	0.581	0.578	0.554

A graphical representation of the data summarized in the previous table (table III, page 25) shows a tendency for a linear relationship between the time of incubation and the amount of inorganic phosphate released while a constant amount of substrate and enzyme were present.
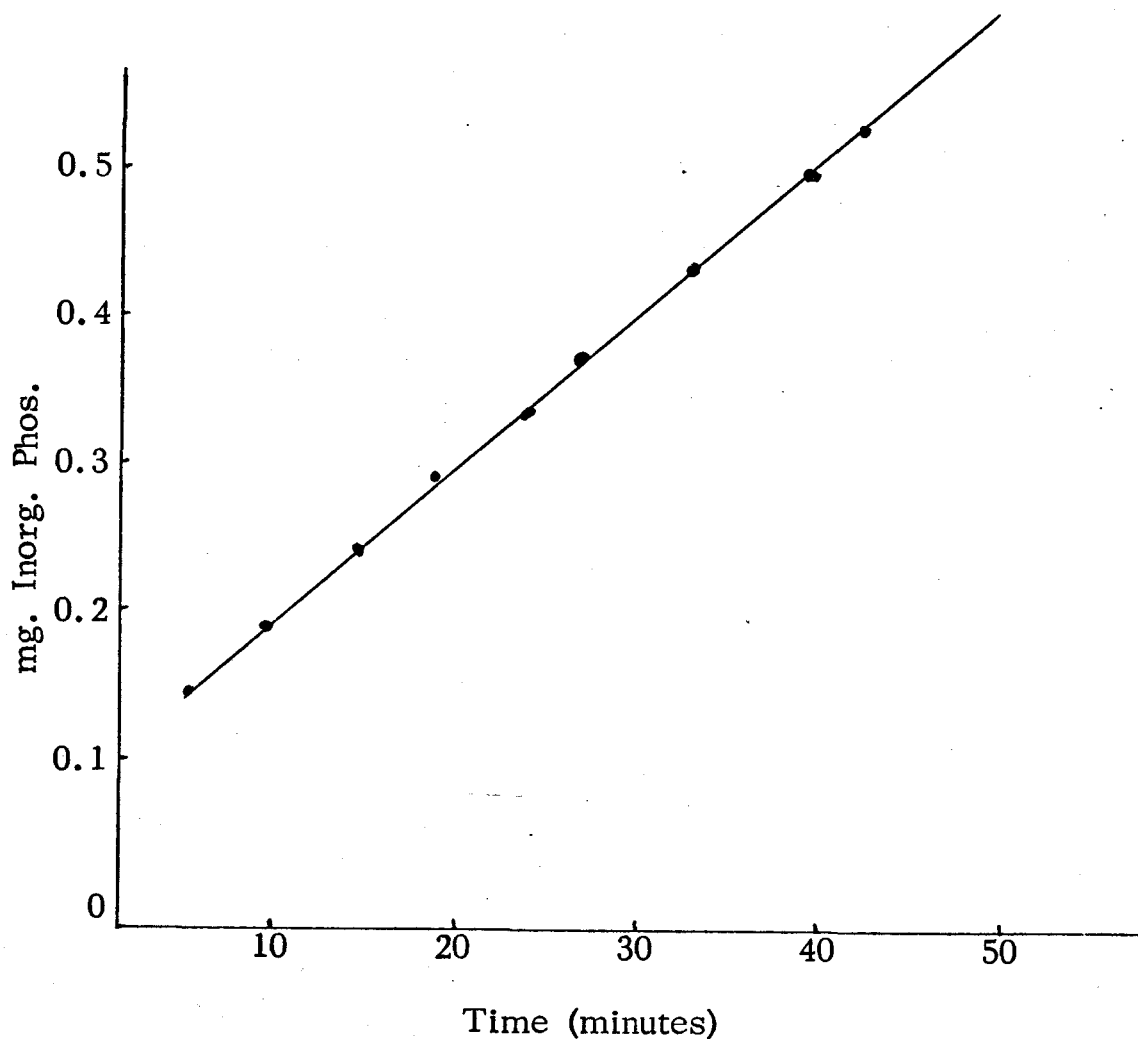

FIG. 3. EFFECT OF TIME

E. EFFECT OF PARATHYROID HORMONE

On the basis of the foregoing studies, it was decided to select those concentrations of both the enzyme and the substrate which were near their optimal levels in order to study the effects of parathyroid hormone on the activity of alkaline phosphatase. The concentrations selected permitted sufficient inorganic phosphate to be liberated in the one hour incubation period such that the levels could be easily evaluated by the method used in this study. A summary of the results are shown in table IV.

TABLE IV

EFFECT OF HORMONE CONCENTRATION

ON HYDROLYTIC ACTIVITY

SAMPLE NO.	ml.* PTH.	mg. INORGANIC PHOSPHATE RELEASED			AVG.
		1	2	3	
1	0.5	0.525	0.490	0.530	0.515
2	1.0	0.530	0.490	0.530	0.517
3	2.0	0.538	0.500	0.534	0.524
4	3.0	0.540	0.510	0.540	0.530
5	4.0	0.548	0.520	0.538	0.536
6	0.0	0.524	0.486	0.530	0.513

* HORMONE CONCENTRATION: 1 milliliter = 1 milligram

The mean values of inorganic phosphate listed in the previous table show no significant differences between each other. It should also be pointed out that the samples appearing in test run number two were conducted in the absence of the magnesium ion. The amount of enzyme activity recorded in test run number two was lower than that seen in runs one and three. This was probably due to the absence of the coenzyme activator, and totally independent of the influence of the hormone on the activity. A graphical plot of the data revealed a curve whose slope was so near zero as not to warrant its inclusion in this section. An eight fold increase in the concentration of the hormone showed an increase of approximately 4 percent in the amount of inorganic phosphate released.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

It was necessary in this study to establish whether or not alkaline phosphatase could hydrolyse the sodium-beta-glycerophosphate substrate in an in vitro experiment, and further, it was necessary to prove the rate of activity of the enzyme. Siffert (50) felt that alkaline phosphatase was physiologically active only in the presence of living cells. If by physiologically active he meant the enzyme was involved in the elaboration of a calcifiable matrix along with certain vital cells, then this study would not disagree with his statement. If, however, the author was saying that the enzyme could hydrolyse a phosphoric acid ester only in the presence of vital cells, this study would strongly disagree with his hypothesis.

The effect of increasing the enzyme concentration in the presence of a constant substrate concentration, resulted in an increase in the amount of inorganic phosphate at the end of one hour. A graph of these results (fig. 1, page 22) showed a linear curve.

When the enzyme concentration was held constant and the substrate concentration increased, the results showed a linear relationship suggestive of the availability of more substrate resulting in an increase in the amount of inorganic phosphate appearing in solution (fig. 2, page 24).

In the time-course study, 10 milligrams of the substrate were used, as it was felt that this amount was sufficient to permit continuous hydrolysis throughout the required incubation period without effecting the maximal activity of the enzyme through excess substrate inhibition. In fact, just slightly more than 50 percent of the theoretically available inorganic phosphate was liberated in the one hour period.

From the results of the aforementioned preliminary studies, relative to enzyme-substrate ratios, it was felt that sufficient substrate had to be available to the enzyme to evaluate the effects of parathyroid hormone on the activity of the enzyme. The 2 milliliters used contained 10 milligrams of substrate and approximately 1 milligram of available inorganic phosphate. The results (table IV, page 27) showed that slightly more than 50 percent of the inorganic phosphate available was released in the control tests without the hormone. The slight apparent increase in activity in those tests in which the hormone was present was not

significant and could in no way account for the great changes in activity

seen in the serum of hyperparathyroid subjects. The activating influence

of the magnesium ion is apparent from the differences in amount of

inorganic phosphate released as shown in table IV. This finding agreed

with that of Kay (26).

Clinical and experimental _in vivo_ studies of hyperparathyroidism

are well documented relative to increased alkaline phosphatase activity

in the serum of these subjects. It seemed quite proper to determine

whether this increased activity was due to a hormonal influence on the

enzyme itself, or if there was just an increase in the concentration of the

enzyme in the serum. From the works of Kay (27) and Klendshoj and

Koepf (29) it was known that parathyroid activity caused a breakdown of

those tissues which were high in alkaline phosphatase concentrations. It

had never been determined, however, whether the hormone could

influence the activity of the enzyme. Chase and Aurbach (13) have

recently shown parathyroid hormone to have an effect on other enzyme

systems. Since the phosphatases have been definitely shown to play an

important part in bone metabolism, one might suspect that parathyroid

hormone could effect the activity of these enzymes. It has been reported

by Weil (58) and Jones and Shinowara (25), that alkaline phosphatase

activity increases with growth.

The results of this investigation show definitely that parathyroid hormone had no significant effect on the hydrolytic activity of alkaline phosphatase _in vitro_. It can only be concluded on the basis of this study, that previously reported increases in alkaline phosphatase activity, relative to hyperparathyroid states, could only have resulted from the breakdown of those tissues sensitive to parathyroid hormone, and containing a considerable concentration of this enzyme. It might be of interest to determine the affects, if any, on acid phosphatase activity _in vitro_ since this enzyme is active in bone destruction.

CHAPTER V
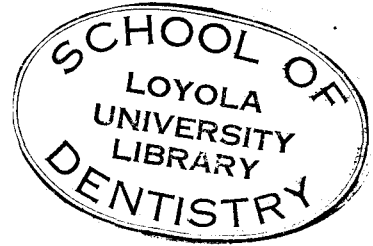
SUMMARY

The results of several preliminary studies involving the activity of alkaline phosphatase on sodium-beta-glycerophosphate were discussed. These included the following experiments:

1. Variation of the enzyme with the substrate constant;

2. Variation of the substrate with the enzyme constant; and

3. A time-course study.

The results obtained were consistent with those reported in the literature for this enzyme.

The results of the effect of parathyroid hormone on alkaline phosphatase clearly shows that the hormone has no activating influence on the enzyme in vitro. The data also supports the hypothesis, found in the literature, that the increase serum enzyme activity in hyperparathyroid conditions is associated with a breakdown of tissues which are high in alkaline phosphatase content, such as bone.

BIBLIOGRAPHY

1. Albright, F., and Ellsworth, R. "Studies on the physiology of the parathyroid gland. I. Calcium and phosphorus studies on a case of idiopathic hypoparathyroidism", J. Clin. Invest., 7: 183-201, 1929.

2. Albright, F., and Reifenstein, E. C., Jr. The Parathyroid Glands and Metabolic Bone Disease: Selected Studies. Baltimore: Williams & Wilkins Co., 1948.

3. Aurbach, G. D. "Isolation of parathyroid hormone after extraction with phenol", J. Biol. Chem., 234: 3179-3181, 1959.

4. Barnicot, N. A. "The local action of the parathyroid and other tissues on bone intracerebral grafts", J. Anat., 82: 233-248, 1948.

5. Bartter, F. C. "The effect of intravenous parathyroid extract on a parathyroidectomized dog", Ann. Rev. Physiol., 16: 429-444, 1954.

6. Beutner, E. H., and Munson, P. L. "Time course of urinary excretion of inorganic phosphate by rats after parathyroidectomy and after injection of parathyroid extract", Endocrinology, 66: 610-616, 1960.

7. Bodansky, A. "Phosphatase studies. I. Determination of inorganic phosphate. Beer's law and interfering substances in the Kuttner-Lichtenstein method", J. Biol. Chem., 99: 197-206, 1962.

8. Bodansky, A. "Phosphatase studies. II. Determination of serum phosphatase. Factors influencing the accuracy of the determination", J. Biol. Chem., 101: 93-103, 1933.

9. Bodansky, A., Blair, J. E., and Jaffe, H. L. "Experimental hyperparathyroidism in guinea pigs leading to ostitis fibrosa", J. Biol. Chem., 88: 629-647, 1930.

10. Buchanan, G. D., Kraintz, F. W., and Talmage, R. V. "Renal excretion of calcium and phosphate in the mouse as influences by parathyroids", Proc. Soc. Exp. Biol. and Med., 101: 306-309, 1959.

11. Carnes, W. H. "The demonstration of an effect of parathyroid extract on bone matrix", Am. J. Path., 26: 736, 1950.

12. Chang, H. "Graphs of parathyroid and other tissues to bone", Anat. Res., III: 23-47, 1951.

13. Chase, L. R., and Aurbach, G. D. "Renal adenyl cyclase. Anatomically separate site for parathyroid hormone and vaso-pressin", Science, 159: 545-547, 1968.

14. Collip, J. B. "The extraction of a parathyroid hormone which will prevent or control parathyroid tetany and which regulates the level of blood calcium", J. Biol. Chem., 63: 395-438, 1925.

15. Collip, J. B., Clark, E. P., and Scott, J. W. "The effect of a parathyroid hormone on normal animals", J. Biol. Chem., 63: 439-444, 1925.

16. Copp, D. H. "The hormone of the parathyroid and calcium hemostasis", in: P. J. Gaillard, R. V. Talmage, and A. M. Budy, eds., The Parathyroid Glands. Chicago: Univ. of Chicago Press, 1964. pp. 73-87.

17. Copp, D. H., Cameron, E. C., Chenney, B. A., Davidson, A. G., and Henze, K. G. "Evidence for calcitonin - a new hormone from the parathyroid that lowers blood calcium", Endocrinology, 70: 639-640, 1962.

18. Gomori, G. "Calcification and phosphatase", Am. J. Path., 19: 197-209, 1943.

19. Greep, R. O., and Kenny, A. D. "Physiology and chemistry of the parathyroids", in: G. Pincus and K. Thimann, eds., The Hormones-III. New York: Academic Press, 1955.

20. Gutman, A. B., and Gutman, E. B. "A phosphorylase in calcifying cartilage", Proc. Soc. Exper. Biol. and Med., 48: 689-691, 1941.

21. Handler, P., Cohn, D. V., and DeMaria, W. J. "Effect of para-thyroid extract on renal extraction of phosphate", Am. J. Physiol., 165: 434-441, 1951.

22. Hanson, A. M. "The hydrochloric X of the bovine parathyroid and its phosphotungstic acid percipitate", Milit. Surg., 54: 76-81, 1924.

23. -----. "Parathyroid preparations", Milit. Surg., 54: 554-560, 1924.

24. Howard, J. E. "Present knowledge of parathyroid function, with especial emphasis upon its limitations", in: G. E. Wolstenholme and C. M. O'Connor, eds., Ciba Foundation Symposium on Bone Structure and Metabolism. Boston: Little, Brown and Company, 1956. pp. 206-218.

25. Jones, L. M., and Shinowara, G. Y. "Serum inorganic phosphate and alkaline phosphatase activity in hypophysectomized rats", J. Biol. Chem., 142: 935-938, 1942.

26. Kay, H. D. "Plasma phosphatase I: Method of determining some properties of the enzyme", J. Biol. Chem., 89: 235-246, 1930.

27. Kay, H. D. "Plasma phosphatase II: The enzyme in disease, particularly in bone disease", J. Biol. Chem., 89: 249-266, 1930.

28. Kleeman, C. R., Bernstein, D., Rockney, R., Dowling, J. T., and Maxwell, M. H. "Studies on the renal clearance of diffusible calcium and the role of the parathyroid glands in its regulation", Yale J. Biol. & Med., 34: 1-30, 1961.

29. Klendshoj, N. C., and Koepf, G. F. "Serum phosphatase activity in hyperparathyroidism", J. Clin. Endocrinol., 3: 351-352, 1943.

30. Kochokian, C. D., and Terepka, A. R. "Effect of parathyroid extract on tissue enzymes and serum and urinary phosphorus", Am. J. Physiol., 165: 142-148, 1951.

31. Kohn, A. "Studien über die schilddrüse", Arch. Mikr. Anat., 44: 366-422, 1895.

32. Lavender, A. R., Pullman, T. N., Rasmussen, H., and Aho, I. "Studies on the intrarenal effects of parathyroid hormone", in: R. O. Greep and R. V. Talmage, eds., The Parathyroids. Springfield, Illinois, Charles C. Thomas, 1961.

33. Löe, H. Bone Tissue Formation: A Morphological and Histochemical Study. Stockholm: A. B. Fahlcrantz Boktryckeri, 1959.

34. MacCallum, W. G., and Voegtlin, C. "On the relation of the parathyroid to calcium metabolism and the nature of tetany", Bull. Johns Hopk. Hosp., 19: 91-92, 1908.

35. -----. "On the relation of tetany to the parathyroid glands and to calcium metabolism", J. Exp. Med., 11: 118-151, 1909.

36. MacIntyre, I., Foster, G. V., and Kumar, M. A. "The thyroid origin of calcitonin", in: P. J. Gaillard, R. V. Talmage, and A. M. Budy, eds., The Parathyroid Glands. Chicago: Univ. of Chicago Press, 1965. pp. 89-102.

37. Mandl, F. "Therapentischer versuch bei ostitis fibrosa generalisata mittels exstirpation eines epithelkörperchentumors", Wien. Klin. Wschr., 38: 1343-1344, 1925.

38. McLean, F. C. "The ultra structure and function of bone", Science, 127: 451-456, 1958.

39. McLean, F. C., and Bourne, G. H., eds. The Biochemistry and Physiology of Bone, Chapter XII. New York: Academic Press, 1956.

40. McLean, F. C., and Urist, M. R. Bone: An Introduction to the Physiology of Skeletal Tissue. Chicago: Univ. of Chicago Press, 1955.

41. Munson, P. L. "Purification of Parathyroid Extract", Fed. Proc., 18: 291, 1959.

42. Munson, P. L., Hirsch, P. F., and Tashjian, A. H. Jr. "Parathyroid Gland", Ann. Rev. Physiol., 25: 325-368, 1963.

43. Neuman, W. F., and Neuman, M. W. "Physiological regulatory mechanism", in: Chemical Dynamics of Bone Mineral. Chicago: Univ. of Chicago Press, 1958. pp. 137-138.

44. Oser, B. J. Hawk's Physiological Chemistry. New York: McGraw-Hill, 1965. pp. 1418-1421.

45. Patt, H. M., and Lockhardt, A. B. "Relationship of a low blood calcium to parathyroid secretion", Endocrinology, 31: 384-392, 1942.

46. Raisz, L. G., Au, W. Y., and Stern, P. H. "Regulation of parathyroid activity", in: P. J. Gaillard, R. V. Talmage, and A. M. Budy, eds., The Parathyroid Glands. Chicago: Univ. of Chicago Press, 1964. pp. 137-152.

47. Robison, R. "The possible significance of hexosephosphoric esters in ossification", Biochem, J., 17: 286-293, 1923.

48. Robison, R., and Soames, K. M. "The phosphoric esterase of ossifying cartilage", Biochem. J., 18: 740-754, 1924.

49. Sandström, I. V. Glandulae Parathyreoideae. Upsala Läk.-Fören. Förh., 15: 441-471; translated by C. M. Seipel with biographical notes by J. A. Hammar, 1938. Bull. Inst. Hist. Med. Johns Hopk. Univ., 6: 179-222, 1880.

50. Siffert, R. S. "The role of alkaline phosphatase in osteogenesis", J. Exper. Med., 93: 415-426, 1951.

51. Sumner, J. B. "A method for the colorimetric determination of phosphorus", Science, 100: 413-414, 1944.

52. Talmage, R. V., and Elliott, J. R. "Removal of calcium from bone as influenced by the parathyroids", Endocrinology, 62: 717-722, 1958.

53. Talmage, R. V., and Kraintz, F. W. "Immediate changes in phosphate excretion following parathyroidectomy in the rat", Proc. Soc. Exper. Biol. & Med., 85: 416-419, 1954.

54. Talmage, R. V., Kraintz, F. W., and Buchanan, G. D. "Effect of parathyroid extract and phosphate salts on renal calcium and phosphorus excretion after parathyroidectomy", Proc. Soc. Exp. Biol. & Med., 88: 600-604, 1955.

55. Talmage, R. V., Kraintz, F. W., Frost, R. C., and Kraintz, L. "Evidence for a dual action of parathyroid extract in maintaining serum calcium and phosphorus levels", Endocrinology, 52: 318-323, 1953.

56. Talmage, R. V., Lotz, W. E., and Comar, C. L. "Action of parathyroid extract on bone phosphorus and calcium in the rat", Proc. Soc. Exper. Biol. & Med., 84: 578-582, 1953.

57. Waldman, J. "Calcification of hypertrophic epiphyseal cartilage in vitro following inactivation of phosphatase and other enzymes", Proc. Soc. Exper. Biol. & Med., 69: 262-263, 1948.

58. Weil, L. "Studies on plasma phosphatase activity during embryonic and tumor growth", J. Biol. Chem., 138: 375-380, 1941.

59. Weinmann, J. P., and Sicher, H. Bone and Bones. St. Louis: C. V. Mosby Co., 1955.

60. Widrow, S. H., and Levinsky, N. G. "The effect of parathyroid extract on renal tubular calcium reabsorption in the dog", Fed. Proc., 21: 435, 1962.

APPENDIX

TABLE V

STANDARD PHOSPHATE CURVE DATA

mg. INORG. PHOS.	OPTICAL DENSITIES				AVG. O.D.
	1	2	3	4	
0.005	0.059	0.056	0.060	0.052	0.057
0.010	0.112	0.111	0.102	0.108	0.108
0.015	0.160	0.160	0.158	0.161	0.160
0.020	0.214	0.210	0.208	0.215	0.212
0.025	0.266	0.260	0.261	0.266	0.263
0.030	0.315	0.320	0.322	0.324	0.320
0.035	0.354	0.359	0.353	0.364	0.355
0.040	0.408	0.414	0.416	0.414	0.413
0.050	0.510	0.515	0.521	0.518	0.515
0.060	0.608	0.617	0.612	0.611	0.612
0.080	0.810	0.810	0.820	0.820	0.820
0.10	1.10	1.10	1.09	1.08	1.09
0.11	1.16	1.20	1.15	1.15	1.16

STANDARD PHOSPHATE CURVE

SAMPLE PREPARED FROM STANDARD
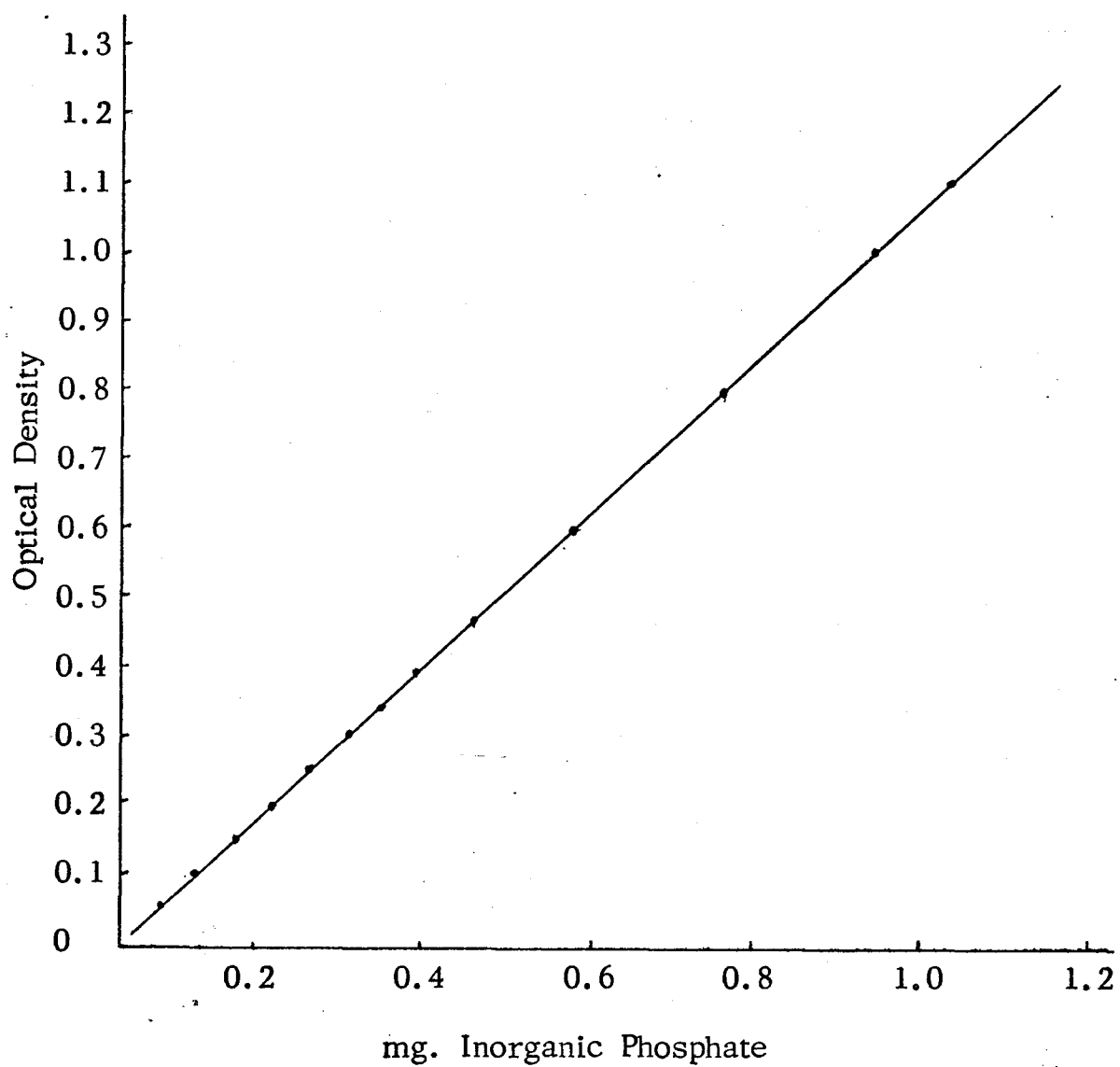
STOCK PHOSPHATE SOLUTION

TABLE VI

S-T CURVE DATA

SAMPLE PREPARED FROM STANDARD

STOCK PHOSPHATE SOLUTION

FILTER 14-213		FILTER 14-214	
WAVE LENGTH	% TRANS.	WAVE LENGTH	% TRANS.
350	8.0	590	64.5
360	12.0	600	62.8
370	18.5	610	61.2
380	25.9	620	59.8
390	32.5	630	58.5
400	39.5	640	57.0
		650	55.6

FILTER 14-214		FILTER 14-215	
400	53.0		
410	61.8	650	58.6
420	67.0	660	57.2
430	72.0	670	55.5
440	75.0	680	54.5
450	77.5	690	53.5
460	78.5	700	53.0
470	78.5	710	53.2
480	78.0	720	53.2
490	77.0	730	53.2
500	76.0	740	53.2
510	74.8	750	53.2
520	73.5	760	53.2
530	72.2	770	53.2
540	70.8	780	52.8
550	70.0	790	52.6
560	68.8	800	52.2
570	67.5	810	52.0
580	66.2	820	51.5

S-T CURVE

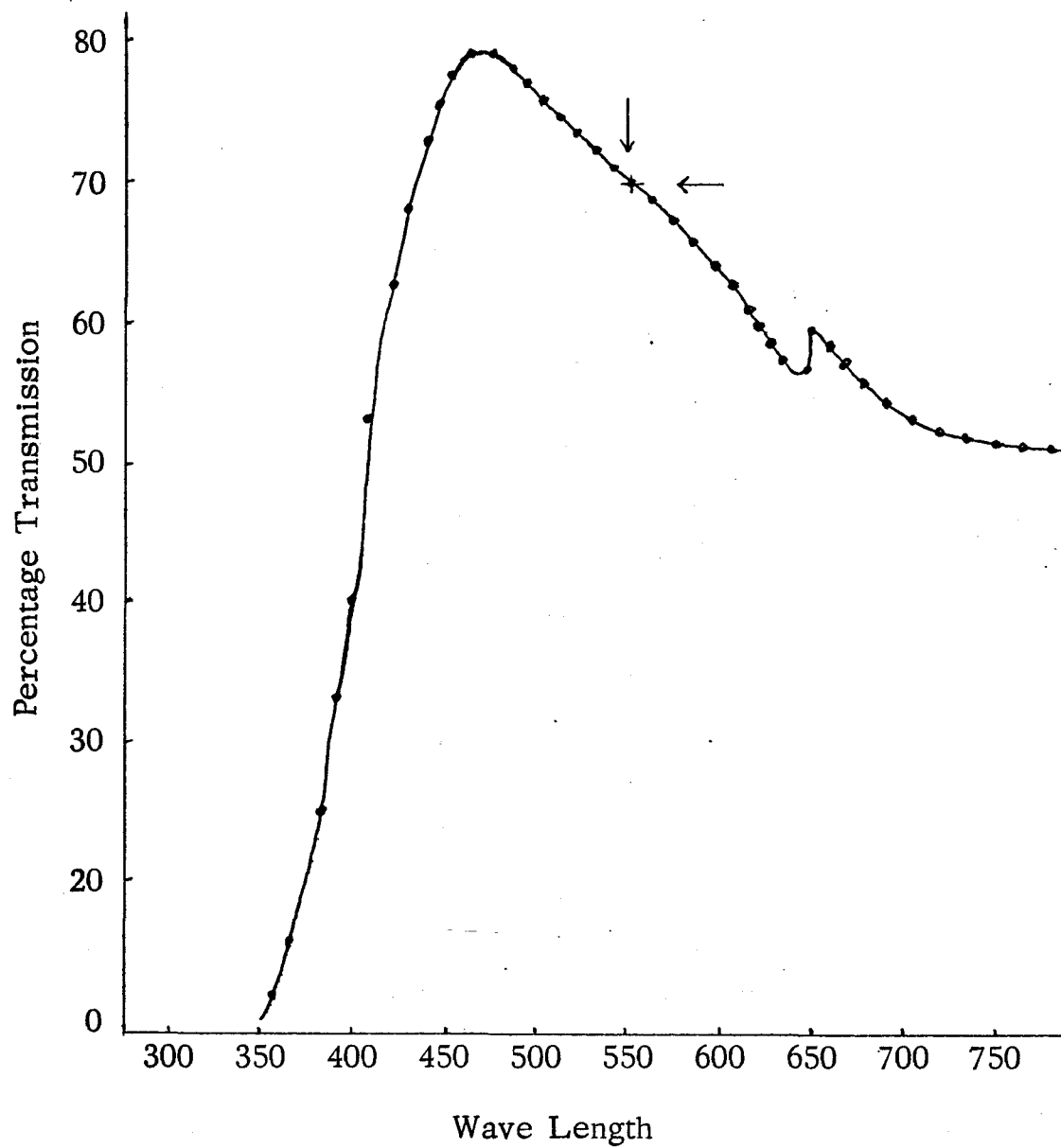
FIG. 5

APPROVAL SHEET

The thesis submitted by Michael Joseph McCormack has been read and approved by members of the Department of Oral Biology.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 22, 1968
Date

[signature]
Signature of Advisor