STUDIES ON THE DETERMINATION OF VITAMIN D

by

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A Thesis

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I. STUDIES ON THE PHOSPHATASE ACTIVITY OF RACHITIC CHICK BLOOD AND THE APPLICATION OF THIS CRITERION IN VITAMIN D BIOASSAYS.

INTRODUCTION

Vitamin D and Rickets.

Rickets is a condition made manifest by defects in the calcification of growing bones (74). The changes in the bones result in outward symptoms which may be recognized clinically, such as, enlargement of the wrists, knees and ankles, bowed legs, and the rachitic rosary.

In normal growth of bone, there appears, on the diaphysial side of the narrow cartilage, a continuous layer of empty cartilage cells forming an almost straight line. Growth of the bone is obtained by the degeneration of a new row of cartilage cells and ossification of the former row. In rickets, the degeneration of the cartilage cells ceases and consequently there is no ingrowth of capillaries and ossification cannot occur. Since this cessation of degeneration does not affect all cells at the same time, the epiphysial cartilage, on the diaphysial side, becomes irregular.

The prevention and cure of rickets may be brought about by the inclusion of vitamin D in the diet provided calcium and phosphorus are also present in adequate amounts. When vitamin D is fed to rachitic animals, ossification begins again in the empty cartilage cells. A new row of empty cartilage cells will generally appear within twenty-four hours of ingestion of the vitamin.

Harris (38) states: "The action of vitamin D is not to act directly on the bones, thereby promoting calcification, but so to alter the body fluids that bone salts may be incorporated into the growing bone." It has been suggested (21) that this mode of action of vitamin D is through the conversion of organic phosphorus into inorganic phosphorus which is thereby

made available for bone formation. Nicholaysen and Jansen (63) found that if adequate available calcium and phosphorus were introduced subcutaneously in rats, normal calcification could take place in the absence of vitamin D but, on histological examination, the lack of the vitamin was manifested by malformed architecture of the bone.

Diagnosis of Vitamin D Deficiency.

With our present knowledge, rickets can be diagnosed with certainty, only by a histological examination of the bones for signs of cessation of degeneration of the cartilage cells. While this is a very valuable method in experimental studies in animals it is of no use in clinical work. X-ray diagnosis of rachitic conditions frequently fails to parallel clinical signs and the interpretation of X-ray plates is subject to considerable human error as has been pointed out by Barnes and Carpenter (7).

A more recent and possibly more practical means of diagnosis has been recognized in the activity of the phosphatase enzyme in the blood. Kay (46) reported in 1930 that the phosphatase activity in the sera of rachitic infants was abnormally high. Bodansky and Jaffe (12) concluded that the activity of the phosphatase of blood serum was a good index of the activity of rickets and also, since the enzyme activity decreased with the onset of healing, a measure of the rate of healing. In this disease, the increase in the phosphatase activity in the blood is perhaps the first definite evidence of the development of the rachitic condition,

appearing before roentgenogram changes in the bone or alterations in the serum phosphate. When vitamin D is administered, the phosphatase activity decreases toward the normal.

Bodansky and Jaffe (12) also reported that the serum phosphatase activity was directly related to the presence or absence of vitamin D. Barnes and Carpenter (7) came to a similar conclusion after studying the phosphatase enzyme activity of the sera of one hundred and eighty-seven infants in Detroit using the technique of Bodansky and Jaffe. In these results, 65.8 per cent of the infants showed active rickets as interpreted from the phosphatase analyses, results which were fairly well in accord with clinical data. X-ray plates of the same infants showed only 26.7 per cent to have active rickets indicating that the X-ray test was a much less sensitive means of diagnosis of vitamin D deficiency.

The various physiological effects of vitamin D deficiency form the basis of several different methods for the biological assaying of the potency of vitamin D carriers.

Methods of Bioassay of Vitamin D Carriers.

A considerable number of methods for the biological assay of vitamin D-potent substances have been devised. Roentgenography (65), line tests as applied to rats (60), and various modifications along similar lines have been tried. Up to the present time, however, the most accurate method for biological assay of vitamin D is believed to be the determination of the ash content of the fat-free, dry bone of the experimental animals, fed on rations to which graded doses of the test substance have been added (52, 64). This latter method is used with chicks since there is some

doubt as to the value of the line test with birds. Healing of rickets in chicks does not show as a line of calcification across the metaphysis of the cut bone but is diaphysial according to McCollum, Orent-Keiles and Day (59).

Recently, McChesney and Homburg (57) have published a modification of the line test which they claim to be applicable to chick vitamin D assays.

The bone ash method, while fairly accurate, is at the same time laborious, time-consuming and expensive. Accordingly, this investigation has been undertaken to determine if there exists a correlation between the degree of rickets in the chick and the level of phosphatase activity in the blood. If such a relation exists, a simpler, more rapid, and less laborious method for assaying the vitamin D potency of a substance could possibly be devised.

History of Phosphatase.

Enzymes hydrolysing phosphoric esters have been known since 1907, when Suzuki, Yoshimura and Takaishi (77) reported the presence in rice and wheat brans, of an enzyme capable of decomposing phytin (the calcium-magnesium salt of inositol hexaphosphoric ester) with liberation of inorganic phosphate. McCollum and Hart (58) in 1908 demonstrated that animal tissues would also hydrolyse phytin. In 1911, Levene and Medrigreceanu (53) found that plasmata from various organs of the dog would hydrolyse nucleotides with the production of free phosphate and in 1912, Grosser and Husler (33) found wide distribution in animal organs of an enzyme capable of hydrolysing glycerophosphate. In the same year, Euler (26) showed that mammalian

intestinal mucosa and kidney extracts would hydrolyse hexose diphosphate, and Harding (37) found that ricinus lipase and emulsin would destroy the same substrate.

Plimmer (70) in 1913, confirmed much of this work and further extended the field of investigation to other phosphoric esters and enzyme preparations. The following year, Harden and Robison (36) commenced the work which was to lead up to Robison's explanation of calcification (72) based on the phosphatase enzyme. His hypothesis seemed to fit the facts much more closely than the theory of specific adsorption of ossifying cartilage put forth by Pfaundler (17) in 1904, and Freundenberg and Györgyi (29) in 1920-1923. The observations of Robison form the starting point of a series of researches which have thrown increasing light on the mechanism of calcification in ossifying tissues.

PREVIOUS INVESTIGATIONS

Up to the present time, very little work has been carried out on the so-called 'alkaline' phosphatase of chick blood. Hall and King (35) reported finding no significant difference between normal and rachitic birds in the phosphatase activity exhibited by extracts of chicken bone, kidney, intestine, aorta, proventriculus, gizzard, large intestine, skeletal muscle or cardiac muscle. Common (22) and Correll and Wise (23) have demonstrated that the serum phosphatase of chick blood increases with the incidence of rickets in chicks. Auchinachie and Emslie (3) however, could find no relation between the vitamin D content of the diet and the plasma phosphatase activity of pullets. Peterson and Parrish (68) have reported increases in the plasma phosphatase activity over the activity of whole blood in pullets and cockerels up to five months of age. They also report (68) on the variation of the phosphatase activity in plasma and whole blood of hens during the cycle of one egg.

All the above mentioned workers used methods for determining phosphatase activity which had been previously established. These and many other methods had been worked out for the investigation of phosphatase activity in the plasma or other tissue extracts of mammals, such as humans, rats and rabbits by Bodansky, A. (11), Bodansky, O. (13), Kay(45), Jenner and Kay (44), Lundsteen and Vermehren (56), King, Haslewood and Delory (50), Cayla (16), Fugita (30), Greenberg et al. (32), Huzita (41), Gutman and Gutman (34), Pelzer (67), Roe and Whitmore (73), and others.

Because of the physiological condition in avians, which in many

respects is different from that found in mammals, it was felt that in the chick blood plasma the relative properties of the alkaline phosphatase might not be the same as those in mammals. A further point in favour of this view was the evidence that optimal conditions for the activity of the alkaline phosphatases from the same type of tissue vary among mammals themselves. Such evidence is seen in the work of Bakwin and Bodansky (4), who showed that the magnesium concentration optimum for rat intestine phosphatase was different from the magnesium concentration optimum for cattle intestine phosphatase. The belief has been expressed and evidence presented (20) that two different alkaline phosphatases may exist in the same organ or tissue.

In the light of these evidences it was deemed advisable to establish the optimal working conditions for the chick blood plasma enzyme before any existing correlation could be studied between the incidence of rickets in chicks and the degree of phosphatase activity in their blood plasma.

EXPERIMENTAL

In the following experiments, unless otherwise noted, White Leghorn chicks were used. They were put on test at one day old. The chicks were divided into pens of twenty, banded, weighed and placed in Jamesway Chick Batteries in a temperature and humidity controlled room. Feed and water were allowed ad libitum. The ration used was the basal diet recommended by the Association of Official Agricultural Chemists (64)* for the biological assay of vitamin D and was composed as follows:

	Per Cent
1. Ground Yellow Corn	58
2. Standard Wheat middlings from western hard wheat	25
3. Crude domestic acid precipitated casein	12
Calcium phosphate (precipitated) B.D.H.	2
4. Iodized salt (0.02% KI)	1
5. Non-irradiated yeast (7% min. N.)	2

To each Kg. of above mixture add 0.2 g. $MnSO_4.4H_2O$.

The crude protein content of this ration was around 21.7 per cent while calcium and phosphorus were about 0.85 per cent and 0.90 per cent respectively.

Graded doses of the vitamin D-potent substances were mixed with this basal ration, the chicks grown for three weeks, weighed and blood samples and tibiae taken for analysis.

* As adopted by Vitamin Discussion Group at Ottawa, Canada, Sept. 12, 1940.

1. Canadian Corn - Toronto Elevators.
2. Maple Leaf Milling Company.
3. Quinte Casein.
4. Canadian Industries Limited.
5. Anheuser-Busch - non-debittered.

Preliminary Method.

The method used in the initial phases of this investigation was a combination of that of Lundsteen and Vermehren (56) and that of Bodansky (13) (14). Ammonium chloride-ammonium hydroxide buffer solutions were used with the concentration of ammonium ion in the buffer solution 0.4 Molar. This buffer was adopted since it was shown by Lundsteen and Vermehren (56) that plasma phosphatase is inhibited more by barbituric acid buffers than by ammonia buffers. Lundsteen (55) also showed that citrate buffers interfered with the determination of phosphorus colorimetrically.

Sodium β-glycerophosphate substrate with additions of magnesium and glycine in the concentrations recommended by Bodansky (13) was used.

The phosphorus liberated on digestion of the substrate with the enzyme at 37°C. was determined colorimetrically following the method used by Lundsteen and Vermehren (56) but substituting amino naphthol sulphonic acid reagent for amidol as the reducing agent in the development of the colour. Colour comparisons were made using the Cenco Sheard-Sanford colorimeter and a standard curve. Determinations were made 30 minutes after the addition of the reducing agent.

INVESTIGATIONS RE. METHOD

Sampling.

In young chicks, Common (22) took the blood samples from the external jugular vein, and in adult fowl, from the wing vein, but no detail is given as to his exact technique. Auchinachie and Emslie (3) took blood from the wing vein in adult fowl using a paraffined needle, while cardiac punctures were used on chicks by Correll and Wise (23). Wiese, Johnson, Elvehjem, Hart, and Halpin (78) in their work on perotic chicks drew blood samples by bleeding directly from the carotid artery into oxalated tubes.

Kay (45) stated that blood samples for phosphatase determinations should be drawn in any convenient way which did not entail the passage of the blood over damaged tissues. Accordingly, in the present work, the heart probe, a technique described by Sloan and Wilgus (75) was compared with a method where the blood passed over damaged tissue, i.e. drawing of arterial blood from the severed neck into oxalated tubes. In this latter method, the neck was skinned, the gullet and veins separated away and the neck severed with a pair of sharp scissors.

All samples were collected in individual glass vials, previously oxalated and dried. The oxalate concentration used to prevent clotting was 0.2 per cent. The use of plasma was decided upon as enabling the use of smaller quantities of blood and greater accuracy in dilutions.

In the comparison of the methods, small variations occurred, and it was concluded that the speed and convenience of removing the blood from the neck made it more advantageous than the heart stab technique.

The data are presented in Table I.

Table I.

Phosphatase Activity of Plasma obtained by the Heart Probe Technique and from the Severed Neck of 21-day-old Chicks.

Chick No.	Phosphatase Activity*	
	Heart Probe	Severed Neck.
1	1.305	1.227
2	1.480	1.370
3	0.580	0.650
4	1.790	1.710
5	1.050	1.137
6	0.772	0.916
Average:-	1.163	1.168

* Milligrams of P liberated by the activity of 1 cc. of blood.

Time of Digestion - 1 hour.

Pooled and Representative Samples.

Correll and Wise (23) drew blood samples of "about 3 cc. from 4 to 5 individuals in an assay group to make a pooled sample of blood, which was considered to be representative of the group".

The results of the analyses of several groups of individuals in an assay group as found in the present work are presented in Table II.

From a consideration of the data mentioned above, it is doubtful if a pooled sample of about 3 cc. from each of 4 or 5 individuals could be considered representative. Auchinachie and Emslie (3) avoided pooled samples entirely, but they were able to do this in all cases, since they worked with adult fowl, and an individual sample was large enough for the required determination.

In the present work, the method used for obtaining pooled samples was as follows:- Individual blood samples were collected in oxalated glass vials and exactly $\frac{1}{2}$ cc. of the blood from each chick in the pen was combined for a representative pooled sample. Where analyses were done on the individual chicks as well as on a pooled sample, the average value of the individual determinations was found to be the same as the value obtained for the pooled sample. The results are presented in Table III.

Table II

Plasma Phosphatase Activity of Individual Chicks whose diet contained varying Amounts of Standard Reference Vitamin D Oil per 100 gms. Feed.

Group I - 0 units of Vitamin D.		Group III - 33.75 units of Vitamin D	
Chick No.	Activity*	Chick No.	Activity*
7015	0.463	4388	0.208
7017	0.205	4363	0.221
7018	0.536	4364	0.184
7019	0.298	4387	0.346
7020	0.560	4381	0.221
7021	0.536	4372	0.222
7022	0.635	4371	0.242
7023	0.636	4369	0.141
7024	0.287	4366	0.114
7025	0.395	Average	0.217
7026	0.668		
7027	0.616		
7028	0.509		
Average	0.488		

Group II - 22.5 units of Vitamin D.	
Chick No.	Activity*
7081	0.219
7082	0.162
7083	0.252
7085	0.548
7086	0.412
Average	0.318

* Activity expressed as mgms. of P liberated by 1 cc. of blood in 15 mins.

Table III

Phosphatase Activity of Plasma from Individual Chicks and of a Pooled Sample

Chick No.	Activity*
7015	0.463
7017	0.205
7018	0.536
7019	0.298
7020	0.560
7021	0.536
7022	0.635
7023	0.636
7025	0.395
7026	0.668
7027	0.616
7028	0.509
Average of Individual Determinations	0.505
Pooled Sample	0.516

* Mgms. of P liberated in 15 minutes by phosphatase in 1 cc. of blood.

Preservation of the Sample.

Common (22), in his technique, allowed the samples to clot spontaneously. These were immediately placed in the refrigerator and the serum was separated by centrifugation the same evening when the analyses were carried out.

Cayla (16) found no change in the phosphatase activity after a contact of 24 to 48 hours between the coagulum and the serum, when the samples were kept in the refrigerator. However, he advocates carrying out the analyses as soon as possible after the separation of the serum from the coagulum.

When Bodansky (11) compared the serum phosphatase activity immediately after separation and after 24 hours in the refrigerator, he found increased activity in all preserved specimens. The average increase in phosphatase activity in the 24 hours was about 10 per cent. The decrease in activity did not follow until 3 to 5 days later. An increase in activity of 15 to 20 per cent occurred when the serum was kept at 37° for 4 to 6 hours. He concludes that, after separation of the serum, it should be kept no longer than 1 hour at room temperature or 3 to 4 hours in the refrigerator, before determinations are made.

Auchinachie and Emslie (3) found that the average increase in phosphatase activity in 14 samples of plasma, stoppered and left at room temperature for 19 hours, was 0.01 unit.

In the present work, the samples were centrifuged in dilute saline solution (0.9%) according to the method of Lundsteen and Vermehren (56) within an hour of sampling. The resulting plasma solution

was preserved in a stoppered flask at 4°C. Analyses, using this preparation, were carried out within two hours of sampling.

The sample, when preserved in the refrigerator as oxalated blood, showed a rapid initial increase in the phosphatase activity followed by a slow decrease. When samples were preserved as plasma solutions, a slow decrease in phosphatase activity usually followed the centrifugation. The data of these experiments are shown in Charts I and II.

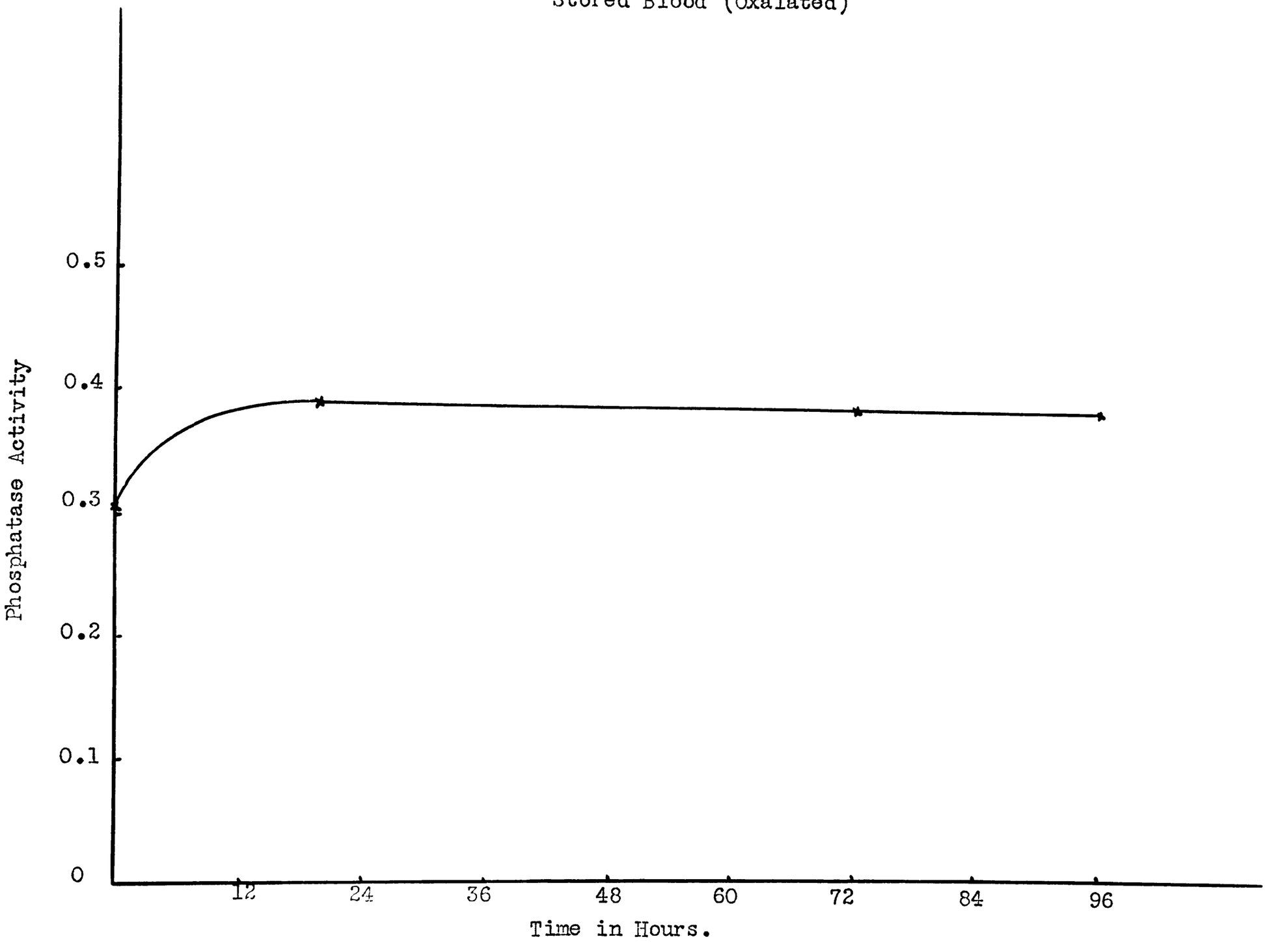
Time of Hydrolysis.

According to the review of Folley and Kay (28), 'much of the confusion in the literature respecting the pH activity optimum of this enzyme, values for which varying between pH 8.4 and 10.0 have been reported, may be traced to the failure of the investigators to determine true initial velocities'. Jacobsen (43) and Folley and Kay (27) have found that the time course of phosphatolysis in its early stages is linear.

The results of a series of experiments regarding the effect of time of digestion are shown in Chart III and these would indicate that the initial velocity is maintained for approximately 25 minutes. The rate of hydrolysis appeared to increase after about 30 minutes, suggesting an induction period and then decreased rapidly. Subsequently, experiments were performed to determine the cause of this induction period. Time curves obtained, using the optimum concentration of magnesium and buffer, namely, 0.05 M and 0.2 M respectively, failed to show the induction period. These data are presented in Charts IV and V.

Chart I.

Variation in Phosphatase Activity of
Stored Blood (Oxalated)



Decrease in Phosphatase Activity of
Stored Plasma Solution.

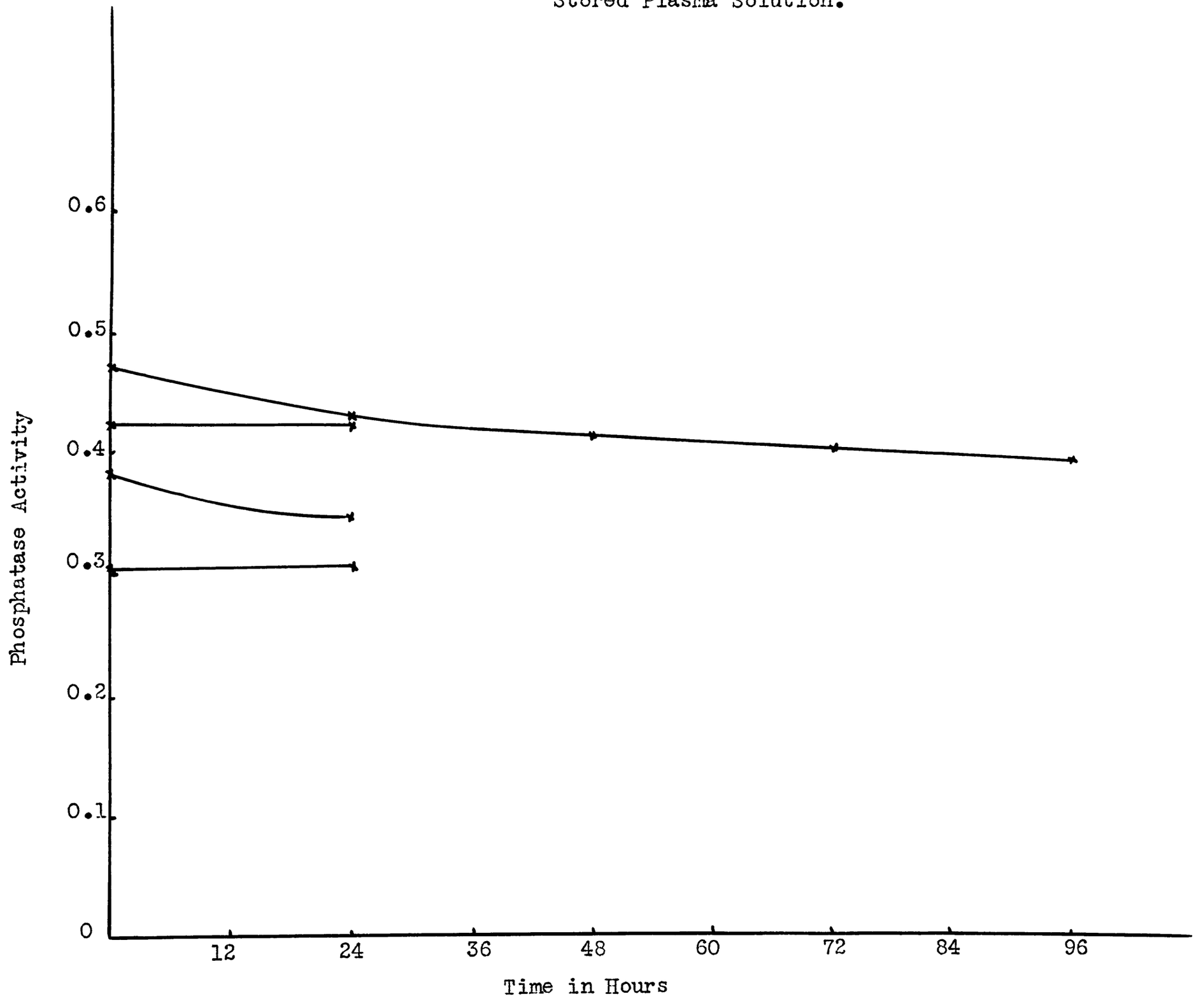
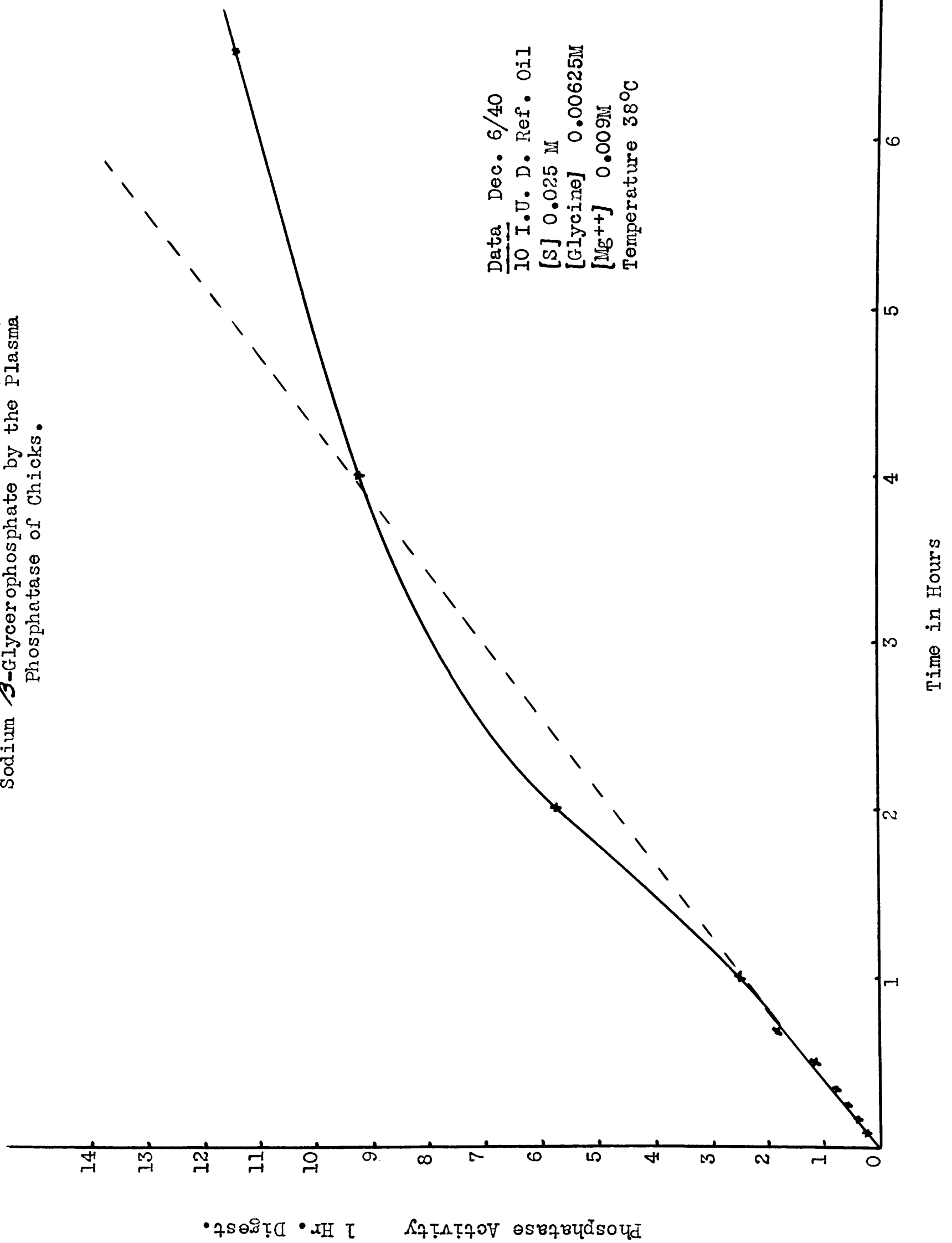


Chart II

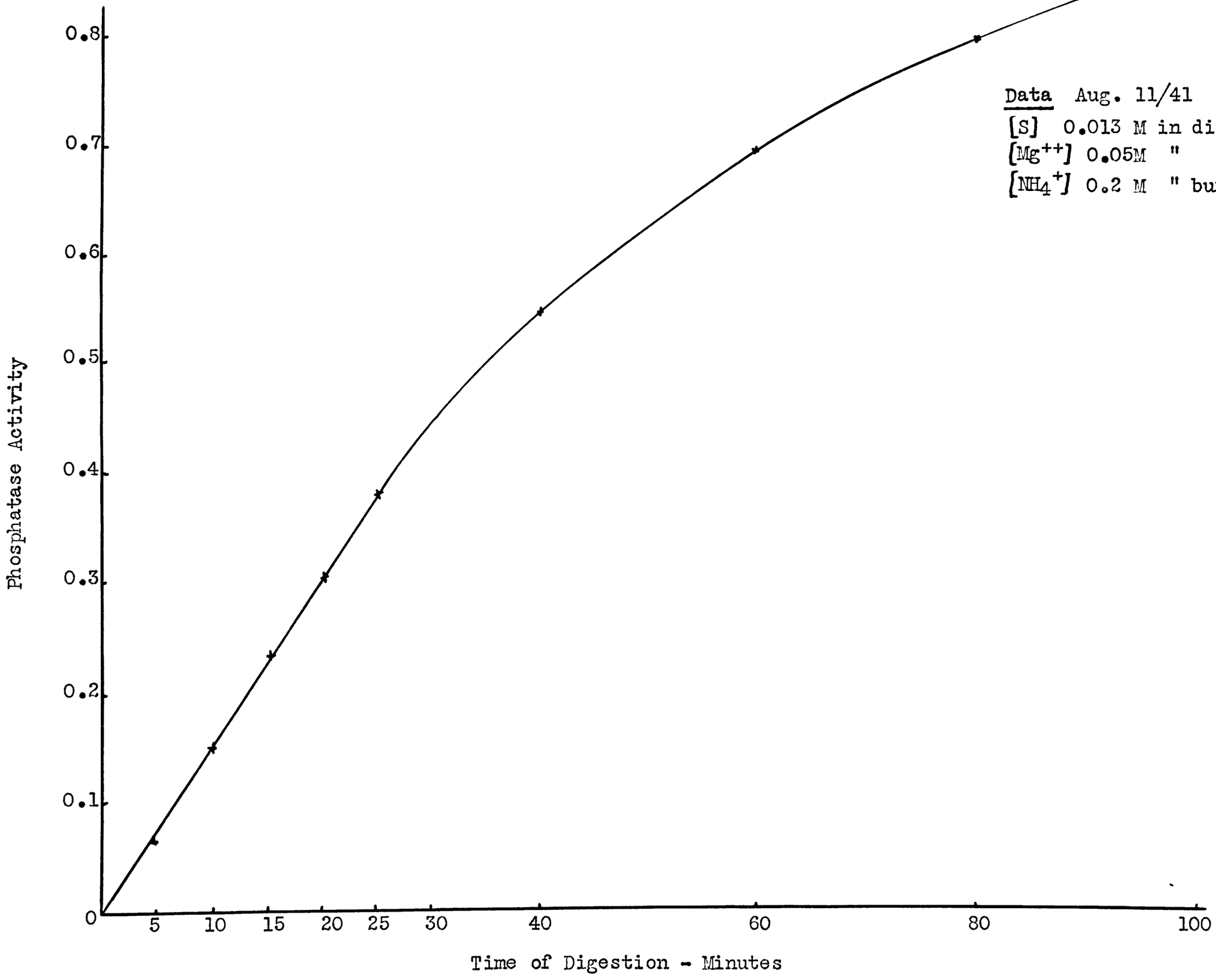
Chart III

Effect of Time on the Rate of Alkaline Hydrolysis of Sodium β-Glycerophosphate by the Plasma Phosphatase of Chicks.



Data Dec. 6/40
10 I.U. D. Ref. Oil
[S] 0.025 M
[Glycine] 0.00625M
[Mg++] 0.009M
Temperature 38°C

Time-Activity Curve for Chick Blood Phosphatase



Data Aug. 11/41
[S] 0.013 M in digest
$[Mg^{++}]$ 0.05M " "
$[NH_4^+]$ 0.2 M " buffer

Chart IV

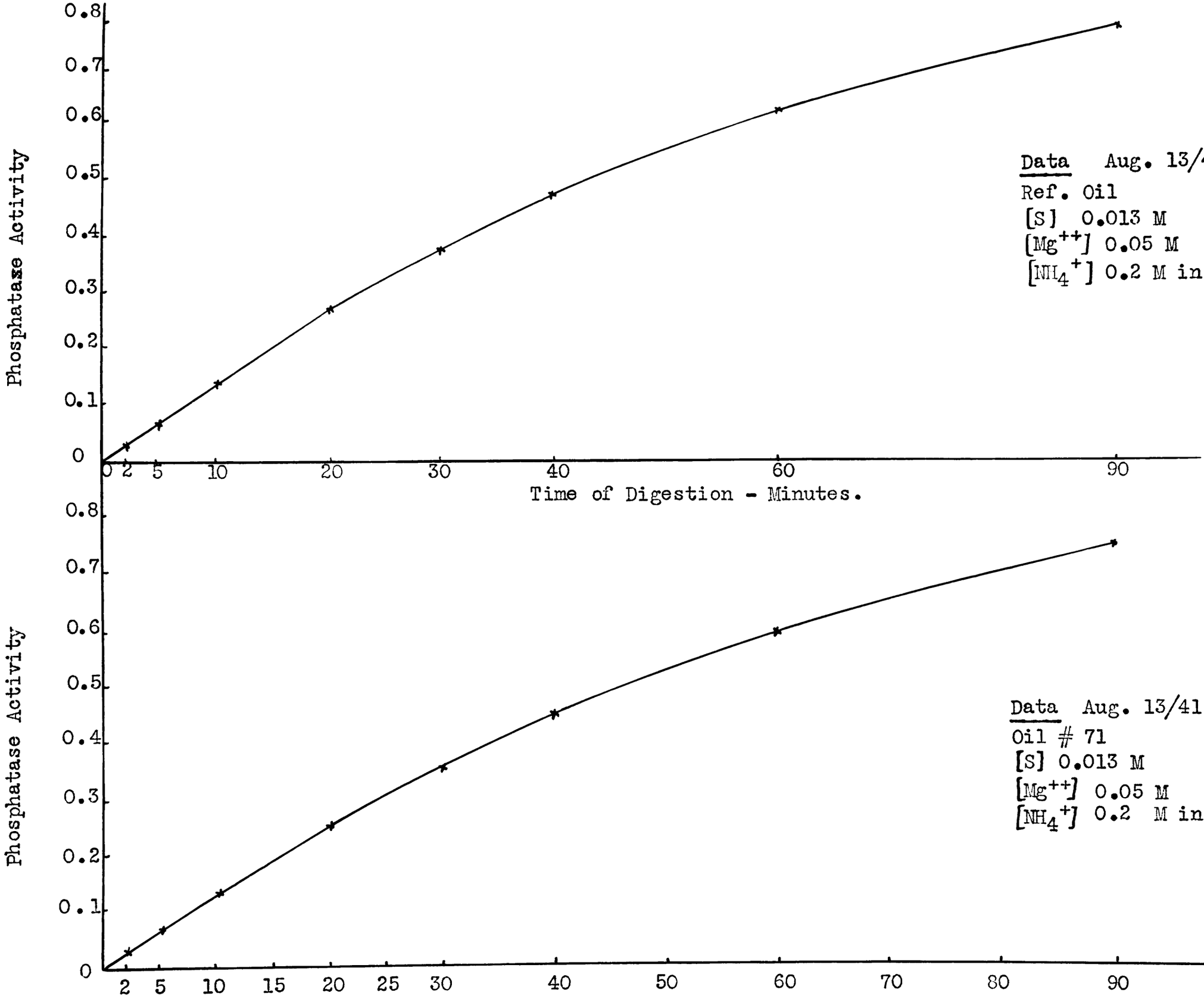

Phosphatase Activity
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 2 5 10 20 30 40 60 90
Time of Digestion - Minutes.
Data Aug. 13/41
Ref. Oil
[S] 0.013 M
[Mg++] 0.05 M
[NH4+] 0.2 M in buffer
Phosphatase Activity
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2 5 10 15 20 25 30 40 50 60 70 80 90
Time of Digestion - Minutes.
Data Aug. 13/41
Oil # 71
[S] 0.013 M
[Mg++] 0.05 M
[NH4+] 0.2 M in buffer

Chart V.

An experiment conducted, using 0.01 M magnesium ion and 0.2 M ammonium ion buffer showed a slight induction period as represented in Chart VI.

Using 0.05 M magnesium ion, 0.2 M ammonium buffer and 0.002 M glycine, the regular type of curve without any induction period was obtained. These data are shown in Chart VII.

When 0.05 M magnesium ion and 0.4 M ammonium ion buffer were used, the induction period showed up very rapidly, no straight line relationship between activity and time being obtained but rather an induction period commencing within the first 10 minutes, as shown in Charts VIII and IX.

From these data, it would appear that the high concentration of ammonium and the low concentration of magnesium ion (0.4 and 0.009 M respectively), which were used in the early part of this work, were responsible for the induction period which occurred in the activity-time curve.

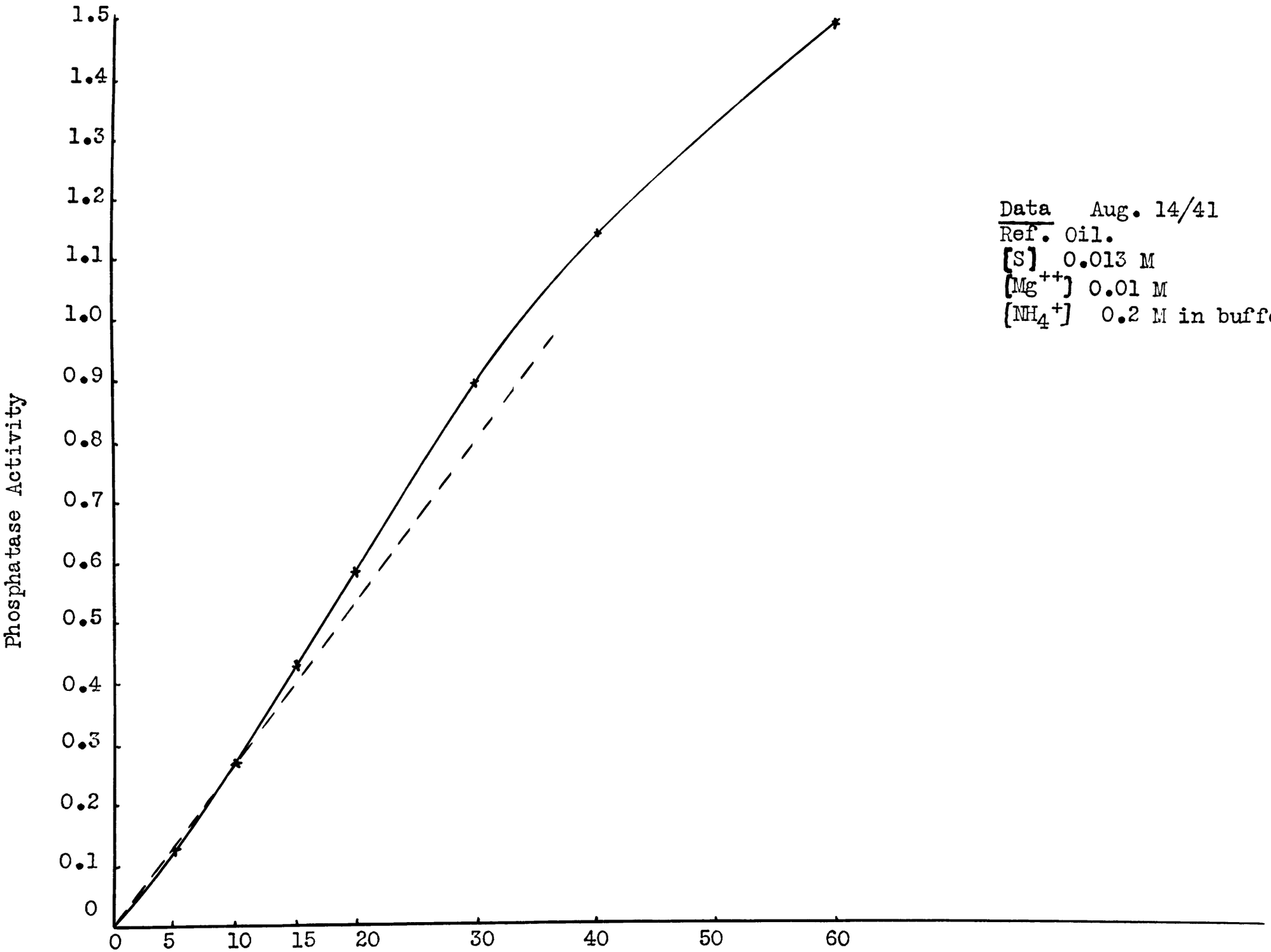
As a further precaution in avoiding this non-linear relationship, the temperature at which the digestions were carried out was reduced to 30°C.

Fifteen minutes was chosen as the time of digestion in order to compare initial velocities in studying the properties of the chick plasma phosphatase relative to its optimum activity under varying conditions.

Optimum pH.

Various pH optima have been ascribed to the phosphatase enzyme depending upon the organ or tissue, the substrate used, its concentration

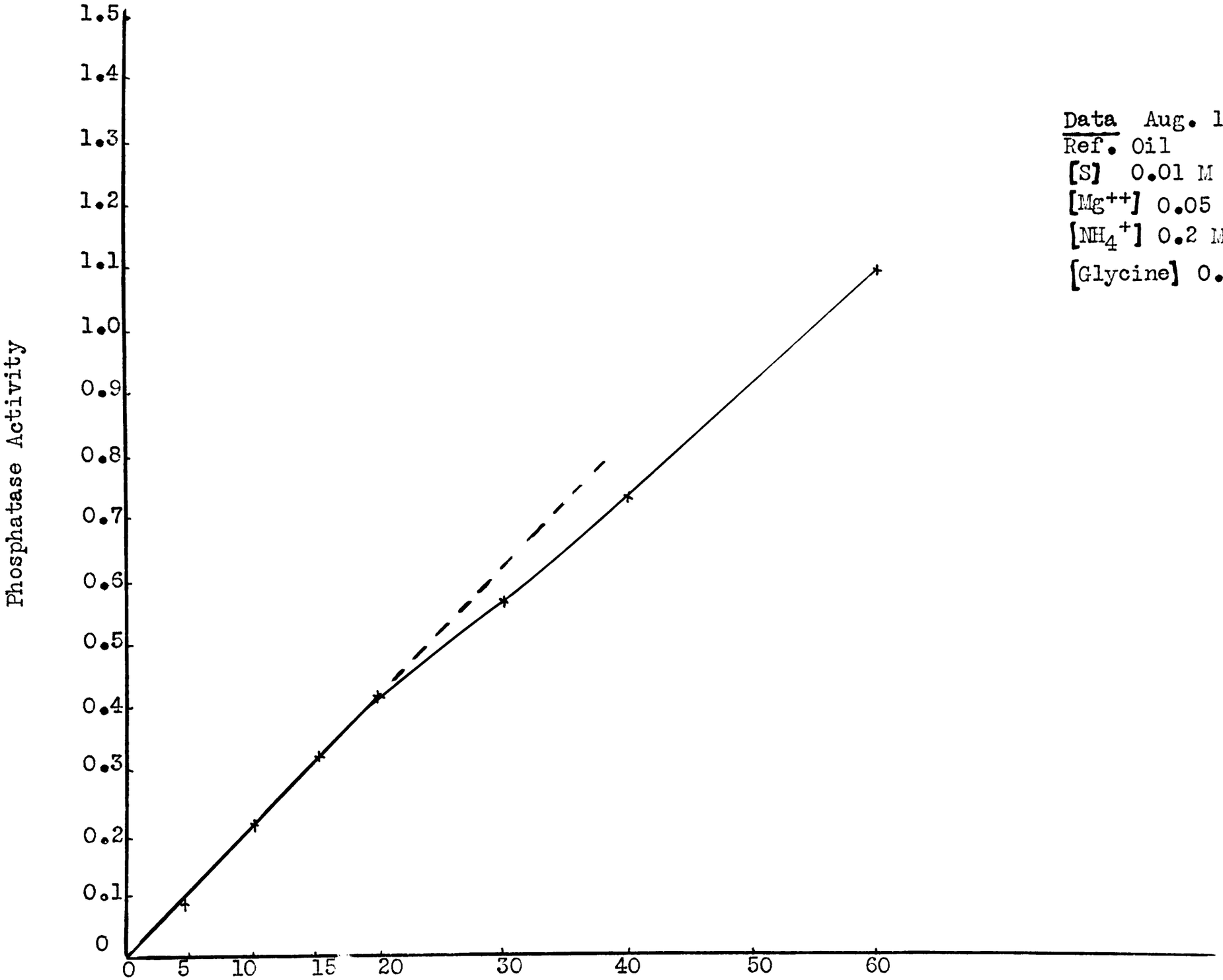
Time-Activity Curve for Chick Blood Phosphatase
Effect of low [Mg^{++}]



Data Aug. 14/41
Ref. Oil.
[S] 0.013 M
[Mg^{++}] 0.01 M
[NH_4^+] 0.2 M in buffer

Chart VI

Time-Activity Curve for Chick Blood Phosphatase
Effect of glycine



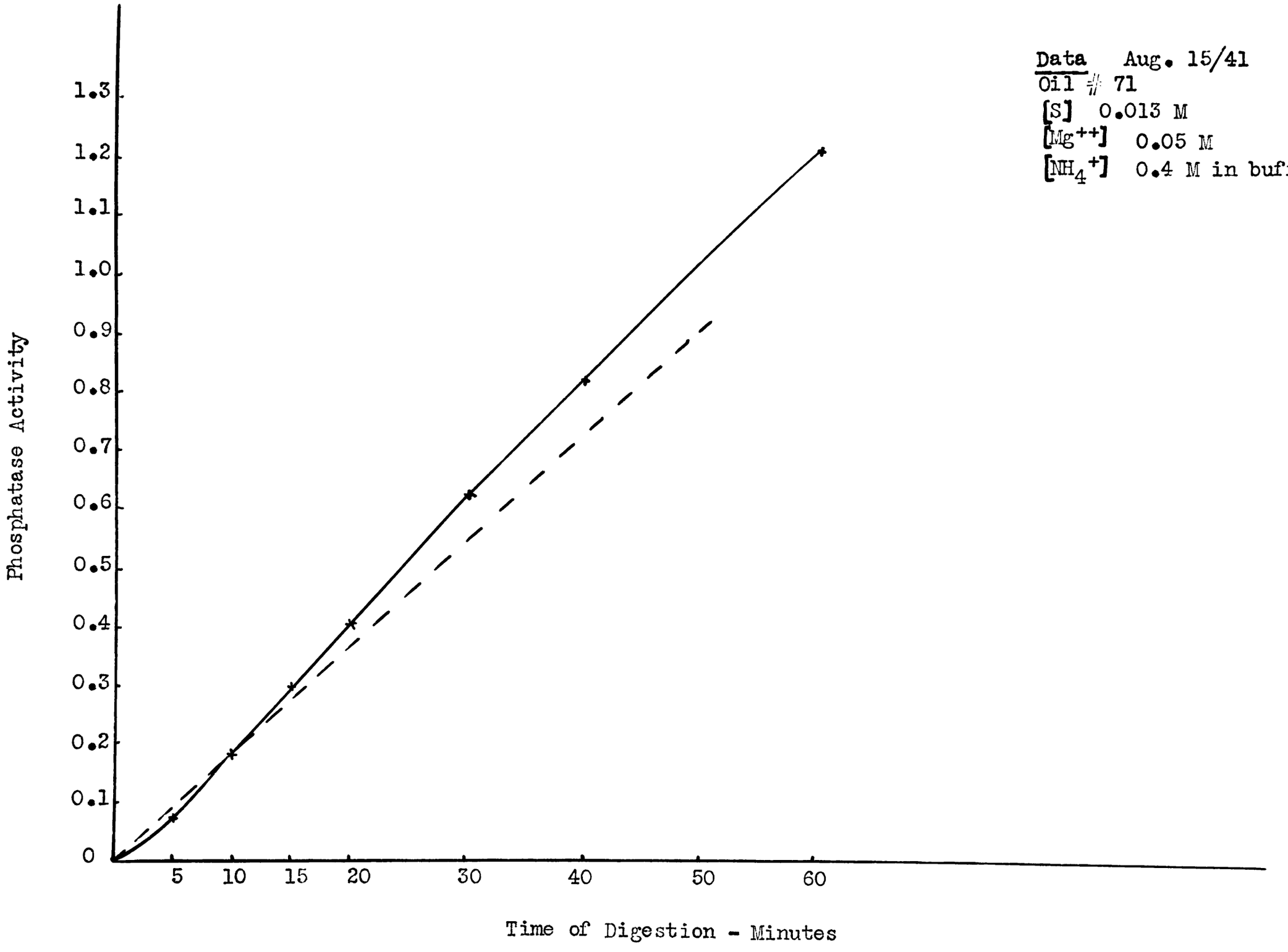
Data Aug. 15/41
Ref. Oil
[S] 0.01 M
$[Mg^{++}]$ 0.05 M
$[NH_4^{+}]$ 0.2 M in buffer
[Glycine] 0.002 M

Chart VII

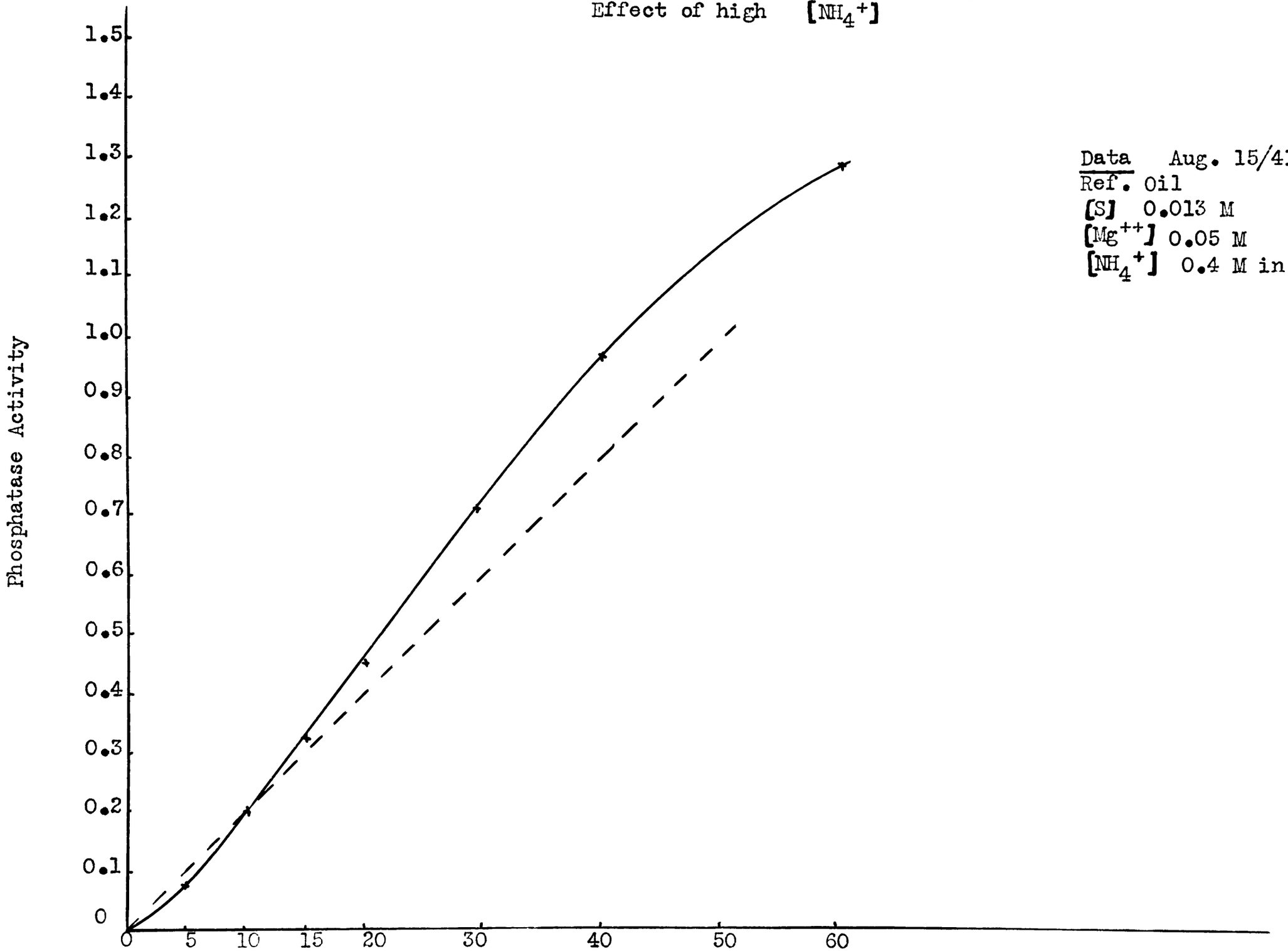
Time-Activity Curve for Phosphatase of Chick Blood
Effect of high $[NH_4^+]$

Chart VIII

Data Aug. 15/41
Oil # 71
[S] 0.013 M
$[Mg^{++}]$ 0.05 M
$[NH_4^+]$ 0.4 M in buffer



Time-Activity Curve for Chick Blood Phosphatase
Effect of high $[NH_4^+]$



Data Aug. 15/41
Ref. Oil
[S] 0.013 M
$[Mg^{++}]$ 0.05 M
$[NH_4^+]$ 0.4 M in buffer

Chart IX

and the time of hydrolysis. (2), (24), (27), (43), (48), etc. Most of these optima lie between pH 8.4 and 10.0 for the alkaline phosphatase.

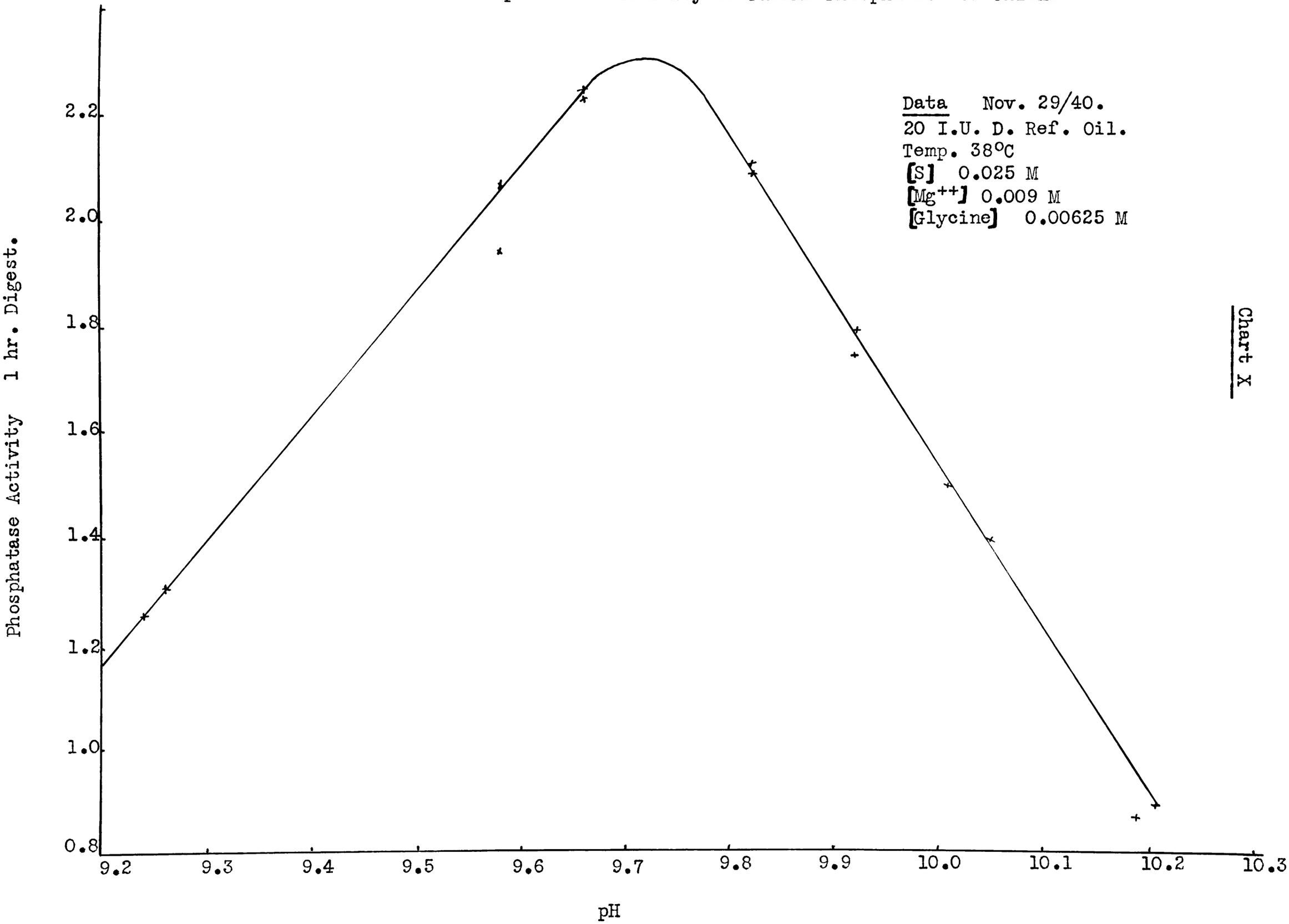
In the case of chick plasma phosphatase, using a 30-minute digestion, 0.025 M sodium β-glycerophosphate and 0.4 M ammonium ion buffer solution, the optimum pH was found to be around 9.7 as shown in Chart X. With the substrate concentration 0.013 M and the digestion period 15 minutes, the optimum was found to be around pH 9.8 as shown in Chart XI. Later, it was found that a concentration of ammonium ion in the buffer solution of 0.2 M was nearly as effective in holding the pH of the reaction mixture and caused less inhibition than a concentration of 0.4 M ammonium ion. These results are shown in Table IV.

Optimum pH was also determined using the carbonate-sodium diethyl barbiturate buffer of King and Delory (49). Under these conditions, however, extensive precipitation of magnesium occurred at pH's higher than 9.5 and this may account for the lower optimum obtained, namely 9.4. This buffer was also found unsatisfactory as to its capacity, there being a considerable pH drop when the blood plasma saline solution was added. The results of this work are shown in Chart XII.

Glycine-sodium hydroxide buffer according to Sorenson (76), gave a much sharper pH optimum at 9.45, but again the precipitation of magnesium occurred at pH's above 9.5. Decreasing the amount of magnesium ion to 0.02 M shifted the precipitation point to approximately pH 9.7 but failed to raise the pH optimum. The results are found in Chart XIII.

In the case of both carbonate-barbiturate buffer and glycine-sodium hydroxide buffer, the optimum activity attained was distinctly less than

Effect of pH on the Activity of Plasma Phosphatase of Chicks



Data Nov. 29/40.
20 I.U. D. Ref. Oil.
Temp. 38°C
[S] 0.025 M
[Mg^{++}] 0.009 M
[Glycine] 0.00625 M

Chart X

Effect of pH on the Activity of the Plasma Phosphatase of Chicks.

Data Mar.13/41.
10 I.U. D. Ref. Oil
Temp. 30°C
[S] 0.013 M
[Mg^{++}] 0.48 M
[Glycine] 0.002 M
0.4M [NH_4^+] in buffer

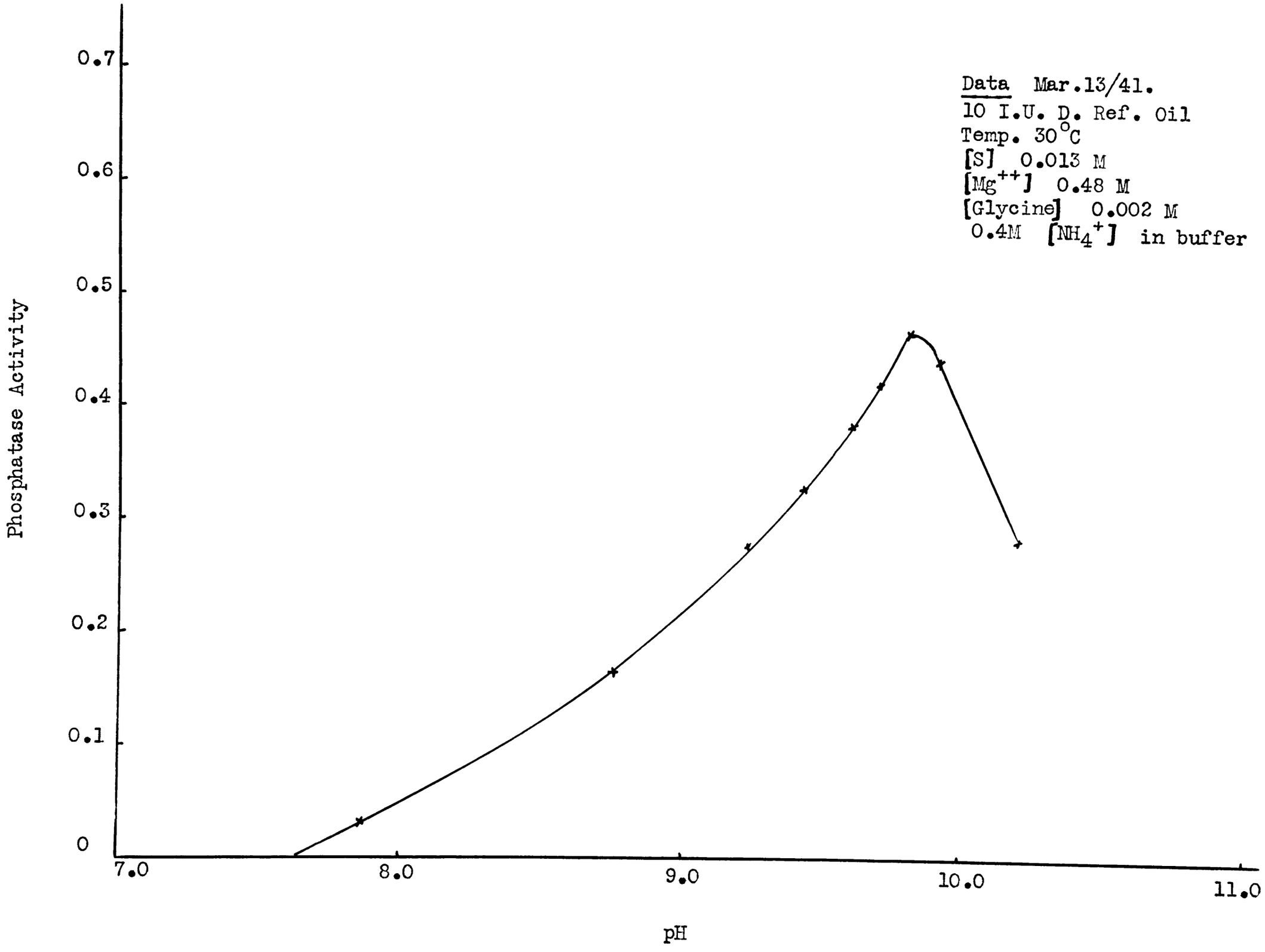


Chart XI

pH Activity Curve
Carbonate - Veronal Buffer

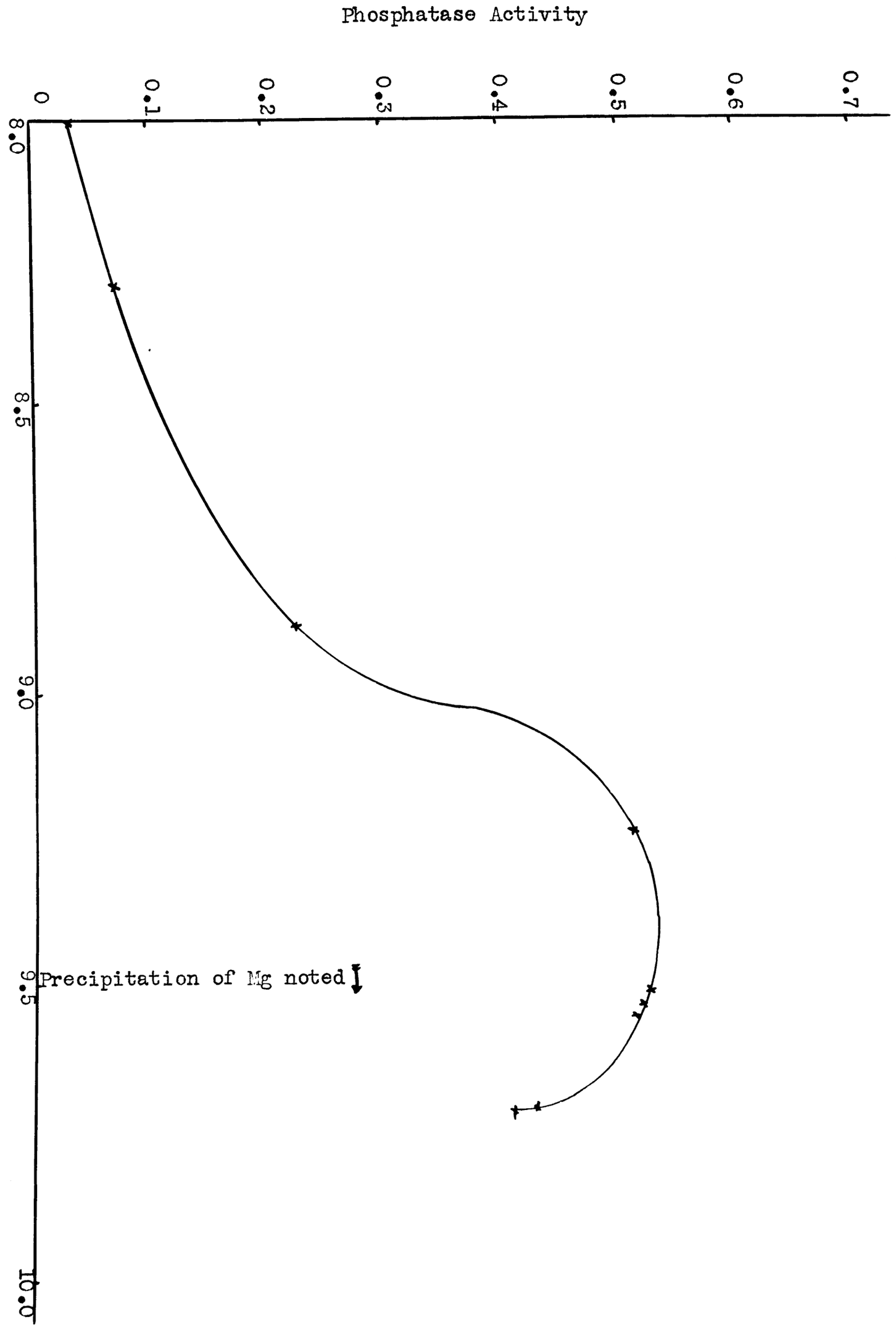
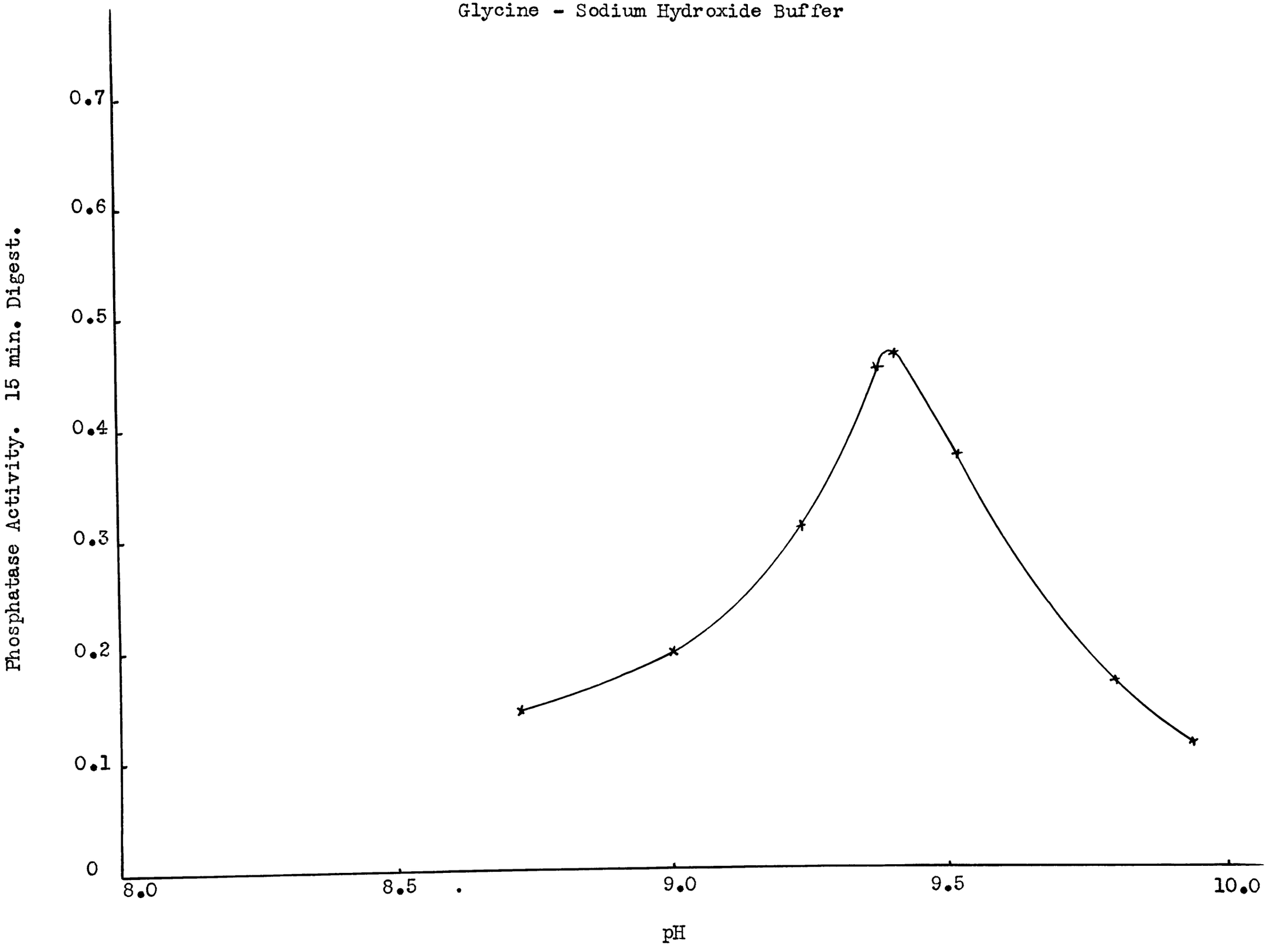


Chart XII

Chart XIII

pH Activity Curve
Glycine - Sodium Hydroxide Buffer



the highest degree of activity using 0.2 M ammonium chloride-ammonium hydroxide buffer at pH 9.75 to 9.8. Comparative figures will be found in Table V. These results would tend to corroborate Lundsteen and Vermehren's statement that barbituric acid buffers caused greater inhibition of phosphatase than ammonia buffers.

Clarke's borate buffer (18) proved too weak to hold the pH of the substrate on the addition of the 0.9 per cent saline. Data are found in Table VI.

The carbonate-hydrochloric acid buffer of Kolthoff (51) caused very heavy precipitation of magnesium thereby destroying both buffer and substrate.

The borax-sodium hydroxide buffer of Sorenson (76) dropped from pH 10.8 to pH 9.85 on addition of the saline at the regular concentration of the buffer. The mixture could not be made up stronger to allow for subsequent dilution because of reaching the limits of solubility of borax.

Bamann and Riedel (5) have shown that the addition of magnesium ion does not alter the pH activity optimum. The high concentration of magnesium ion which could be used in conjunction with ammonia buffer solutions is due to the increased solubility of magnesium hydroxide in the presence of ammonium ion.

At pH 9.0 in the absence of ammonium ion, magnesium ion can be present to the extent of 0.05 M before precipitation of magnesium hydroxide would occur. At pH 9.2, the optimum used by Bodansky, precipitation would occur when magnesium ion was present in concentrations exceeding 0.025 M. This would only be theoretically true and precipitation actually occurs at about 0.02 M concentrations. Hence, Bodansky

would find his optimum very near the limits of solubility of magnesium.

At the pH optimum used in this work, around 9.75, the maximum amount of magnesium ion which could be present in solution under ordinary conditions would be 0.0015 M. In actual practice, no precipitation occurred until this concentration was increased to about 0.5 M. This is due to the presence of ammonium and chloride ions with which Mg^{++} unites to form $MgCl_2.NH_4Cl$ which is readily soluble and would exist in solution in ionic form.

All pH determinations were made with the Coleman glass electrode.

Table IV.

The Effect of Ammonium ion on the Buffer Capacity and Phosphatase Activity.

Conc. of Ammon. ion in buffer.	Conc. of Ammon. ion in Digest.	pH before Digestion	pH after Digestion	Phosphatase Activity (mgms.)
0.2	0.05	9.88	9.80	0.551
0.4	0.10	9.85	9.82	0.443
0.8	0.20	9.83	9.82	0.296

Table V.

The Effect of Barbiturate and Glycine Buffers on Phosphatase Activity as Compared with Ammonia Buffer.

Buffer	Activity at optimum	Buffer	Activity at optimum
Carbonate-Veronal	0.523	0.2 M Amm.	0.657
Glycine-sod. Hydroxide	0.466	0.2 M Amm.	0.492

Table VI.

Clarke's Borate Buffer Solution.

Concentration	pH before adding saline	pH after
Regular	9.80	9.3
2 x Regular	10.05	9.5
4 x Regular	10.00	9.6

Omission of the potassium chloride from the buffer mixture failed to make any improvement.

Regular	10.00	9.43
2 x Regular	10.15	9.62

Optimum Substrate Concentration.

According to enzyme kinetics, maximum activity is effected when the enzyme is saturated with the compound it hydrolyses, that is, when practically 100 per cent of the total enzyme is in combination with the substrate. This is the theory which explains why the activity of the alkaline phosphatase increases with increasing concentrations of sodium β-glycerophosphate until an optimum concentration of the substrate is reached. Further increases in substrate concentration may cause a suppression of the activity. Evidence in support of this theory has been presented by Jacobsen (42), Bamann and Riedel (5), Folley and Kay (27) and others, and has also been noted in the present work as is shown in Charts XIV and XV.

In these charts, it may also be seen that the variations in the phosphatase activity of the plasma of chicks on the same diet, were as great as 75 per cent from the mean and it may be argued that substrate concentrations, that did not give a saturation of the enzyme in one case, may have been above the optimum in another case. Since there is an inhibiting effect due to excess substrate, the true initial velocity, under such conditions, cannot be measured.

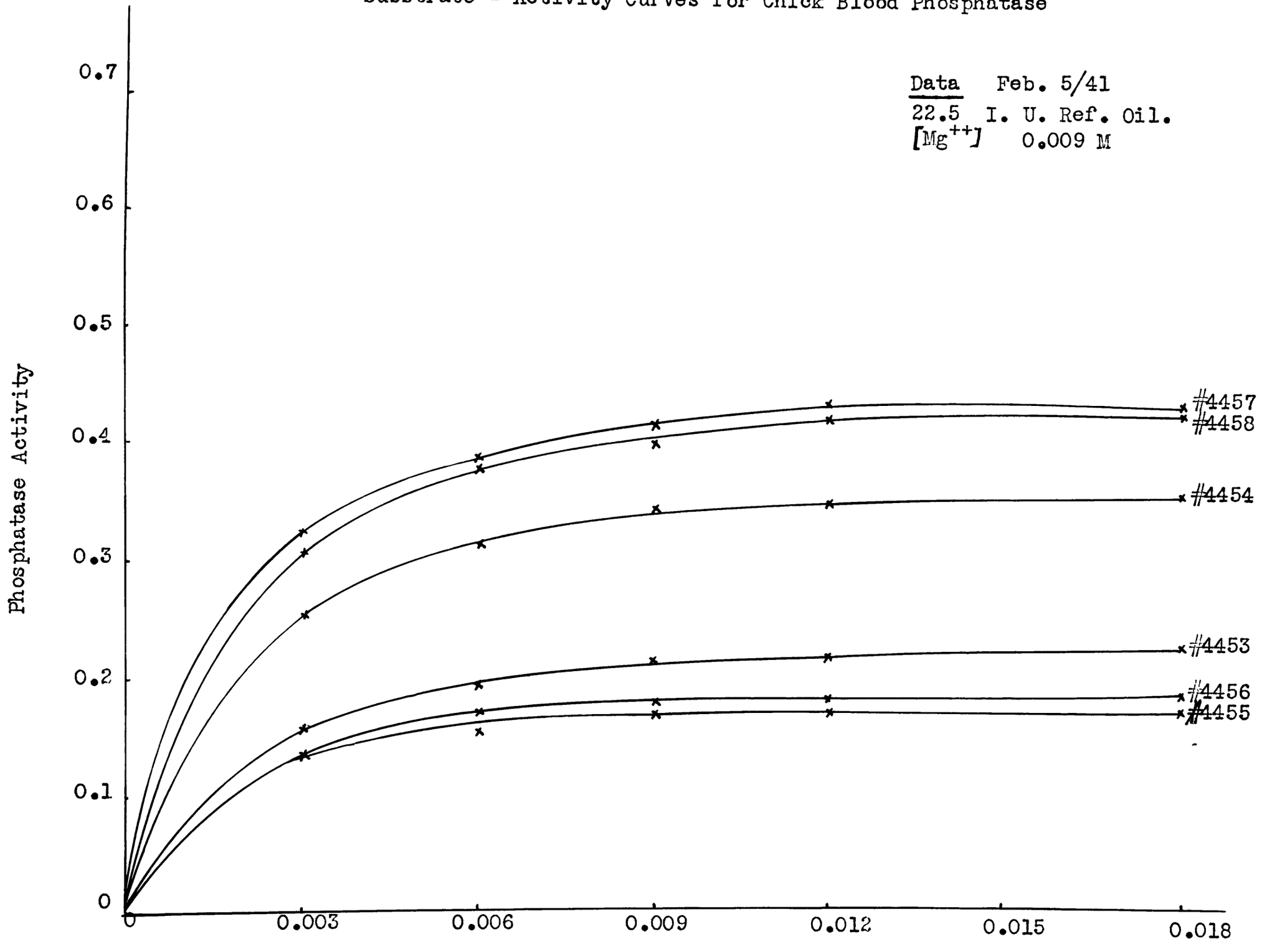
Suboptimal concentration of substrate, on the other hand, would result in the measuring of the activity of the enzyme at different degrees of substrate saturation, and in a pooled sample of chick plasmas, the activity would be conditioned by the degree of saturation of the individual components.

The concentration of Na β-glycerophosphate in the reaction mixture which was finally adopted was 0.013 M, and is somewhat below the optimum

Chart XIV

Substrate - Activity Curves for Chick Blood Phosphatase

Data Feb. 5/41
22.5 I. U. Ref. Oil.
$[Mg^{++}]$ 0.009 M



M concentration of substrate in digest.

Substrate - Activity Curves for Chick Blood Phosphatase

Data Feb. 4/41
10 I. U. Ref. Oil
$[Mg^{++}]$ 0.009 M

#4416
#4411
#4413
#4423
#4419

Phosphatase Activity

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

0 0.003 0.006 0.009 0.012 0.015 0.018

M concentration of substrate in digest.

Chart XV

concentration in nearly all cases. (Under these conditions rarely more than 5 per cent of the total phosphate in the digestion mixture is liberated in the 15 minute period of enzyme activity.)

These difficulties would render quantitative comparisons a matter of some uncertainty and it is with the above considerations in mind that this study must be interpreted.

Activation and Inhibition of Phosphatase by various Metallic Ions and Organic Compounds.

A great deal of contradictory evidence is to be found in the literature regarding the role of various metallic ions and organic compounds as activators and inhibitors of 'alkaline' phosphatase. Magnesium ion has received more attention than any other substance. Most investigators agree that this ion activates the enzyme although the optimum concentrations in the reaction mixtures which are recommended vary over a wide range depending on the substrate and on the type and purity of the enzyme preparation used. Studies on the problem have been made by Albers (1), Bamann and Salzer (6), Bodansky, O. (13), Cloetens (19), Giri (31) and many others. Belfanti et al. (9) claim that magnesium ion does not activate bone phosphatase and Cattaneo et al. (15) discovered only slight activation by magnesium. Wiese et al. (78) found that the blood and bone phosphatases of perotic chicks were activated much more by manganese ion than magnesium ion. Practically all other workers obtained activation of the phosphatase when magnesium ion was added to the substrate. Albers (1) states that magnesium ion is the only activator of purified alkaline phosphatase.

Bodansky, O. (13), working with bone, and Hove (40), working with intestine, maintain that amino acids activate phosphatases. On the other

hand, Albers (1) maintains that amino acids do not activate alkaline phosphatases, and Pyle (71) lists a specific instance where kidney phosphatase was inhibited by Cystine.

Giri (31) and Pyle (71) list ascorbic acid as an inhibitor of phosphatases, Giri working with the plant enzyme and Pyle with that of the kidney. Albers (1) and King and Delory (47) record no activation of phosphatase by ascorbic acid.

Extensive work has been done on other substances such as sodium, cyanide, fluorine, iron, copper, oxalate and many others. Chief among the workers in this particular field are Cloetens (20), Pyle and coworkers (71), Bauer (8) and Albers (1).

In this work, attention was given to magnesium ion, manganese ion, glycine, oxalate, sodium chloride and hemolysis products as possible activators or inhibitors.

Optimum Magnesium Ion Concentration.

To determine the optimum amount of magnesium to add to the substrate for maximum activation, two different methods of treating the plasma were used. Some plasma was dialyzed at 4°C for 24 hours in Visking casings against three changes of distilled water. A precipitate of a protein nature was produced and this was soluble in sodium chloride solution. This precipitate is probably similar to that noted by Cedrangolo (17) and by Bodansky (14). There was no difference in the phosphatase activity between the preparation from which the precipitate was centrifuged off and the one in which the precipitate was dissolved in saline as shown in Table VII. However, when no sodium chloride was added after dialysis, the activity was definitely lower. Where comparisons

were made between the activity of dialyzed and undialyzed plasma solution, the sodium chloride concentration was adjusted to the level of 0.9 per cent in each case. With dialyzed plasma, the optimum magnesium ion concentration ranged from 0.1 M to 0.2 M (qMg 1 to 0.7)*, as shown in Chart XVI. Higher concentrations of magnesium ion caused a precipitation of magnesium hydroxide and consequently a destruction of the buffer.

With undialyzed plasma, the optimum magnesium ion concentration ranged from 0.05 M to 0.2 M (qMg 1.3 to 0.7), also shown in Chart XVI. This is considerably higher than the optimum range described by Kay (44).

Table VII

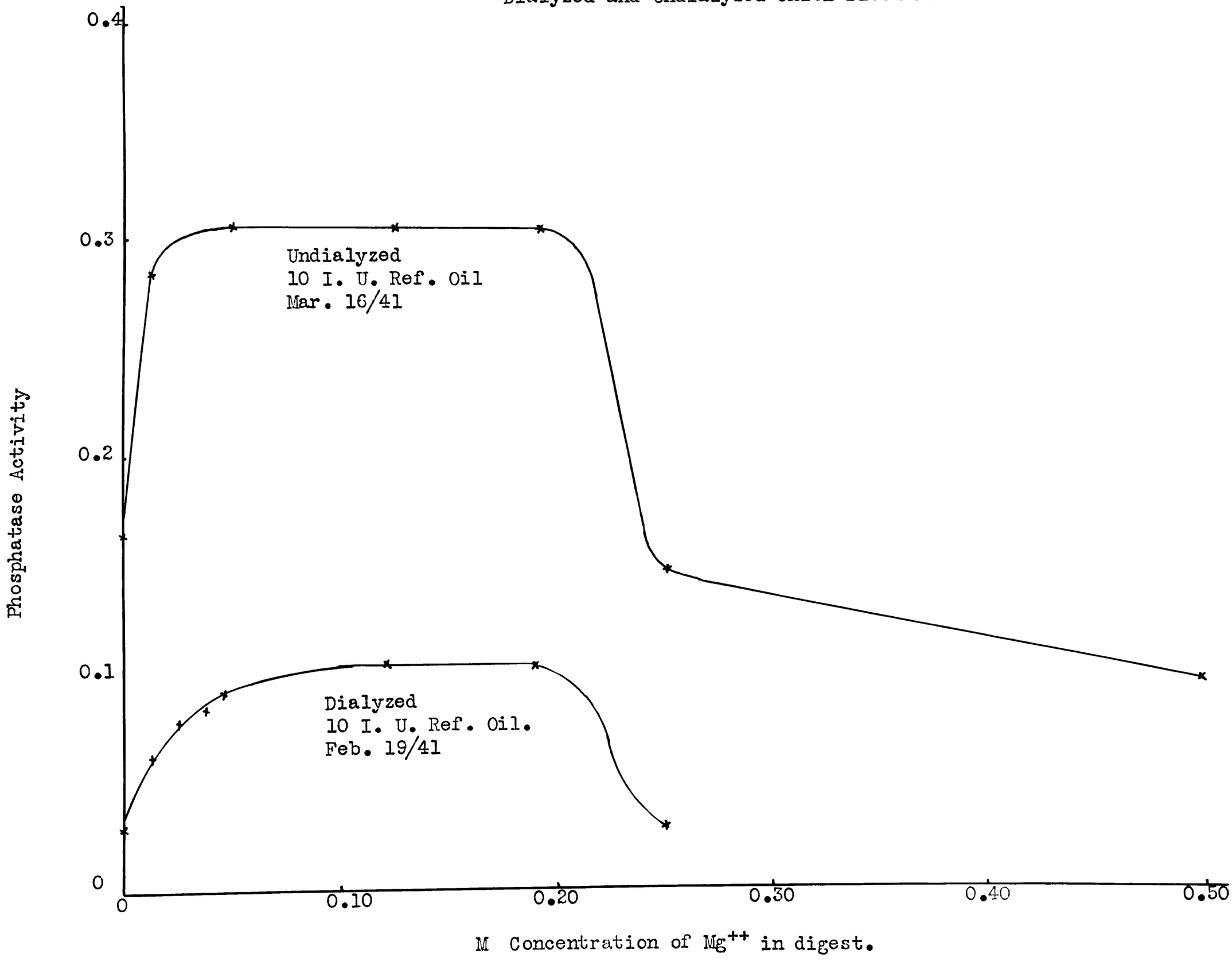
The Effect of Sodium Chloride and the Removal of the Precipitate from the Dialyzed Plasma on the Phosphatase Activity.

Treatment of the Dialysate	Activity. (mgms.)
Not centrifuged. Conc. NaCl in dialysate 0.90%	0.152
Centrifuged. Conc. NaCl in dialysate 0.90%	0.152
Not centrifuged. Conc. NaCl in dialysate 0.45%	0.136
Centrifuged. Conc. NaCl in dialysate 0.45%	0.134

* Negative logarithm of the molarity of magnesium ion in the reaction mixture, as used by Kay (44).

Chart XVI

Effect of Magnesium on the Phosphatase Activity of
Dialyzed and Undialyzed Chick Blood Plasma



Optimum Manganese Ion Concentration

Wiese et al. (78) in their work on phosphatase in perotic chicks found greater activation by manganese ion than by magnesium ion. This did not prove to be the case in the phosphatase of rachitic chicks under the conditions of this experiment. Results are shown in Chart XVII.

In undialyzed plasma, concentrations of manganese ion up to 0.004 M in the reaction mixture produced some activation but not as much as magnesium. Higher concentrations caused very heavy precipitation of manganese hydroxide and consequent destruction of the buffer. In dialyzed plasma, the same optimum concentration of manganese ion was found as shown in Chart XVIII.

Optimum Glycine Concentration.

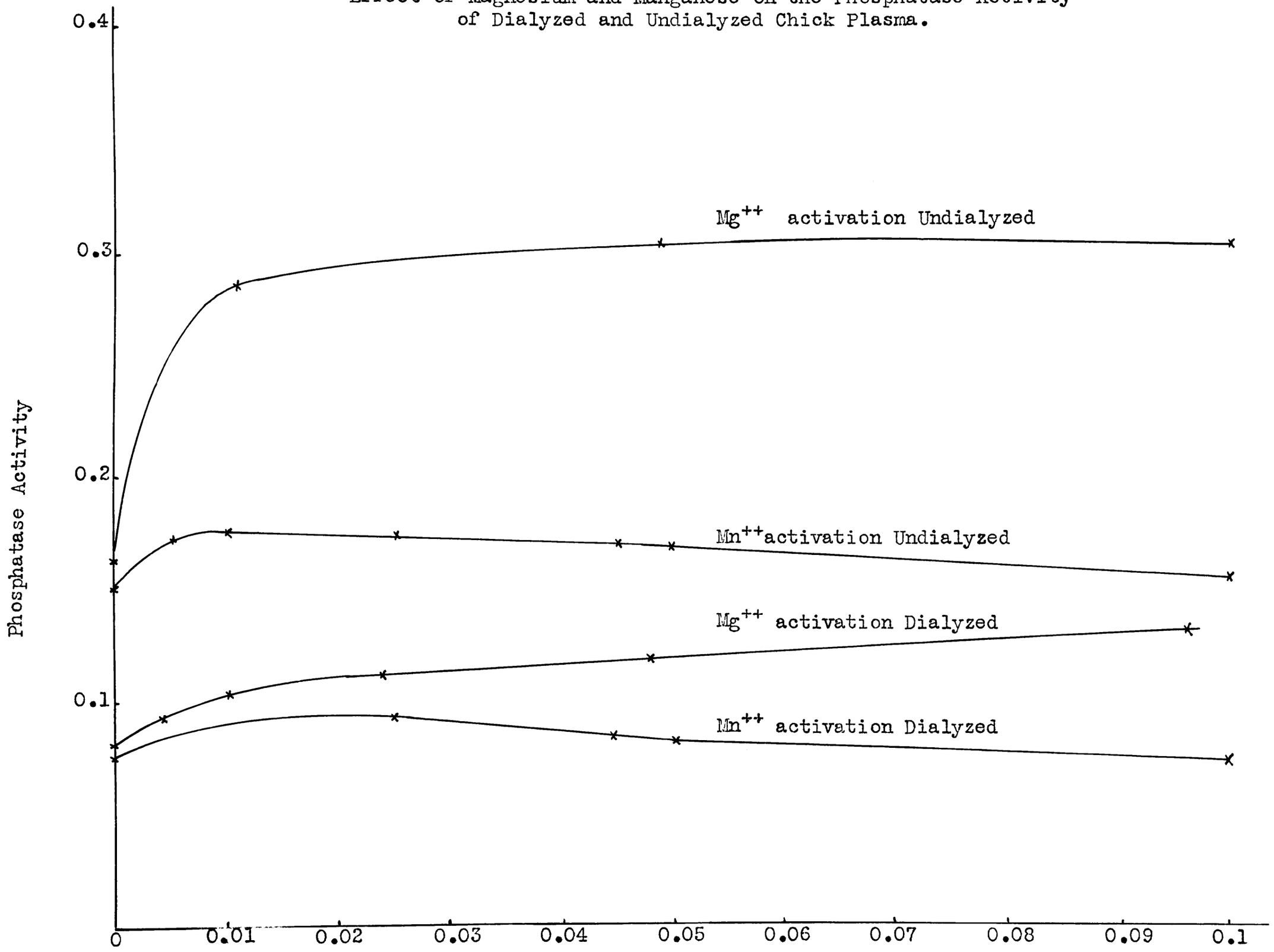
According to Bodansky (13), α-amino acids were effective in activating plasma phosphatase and also brought about a linearity of enzyme activity relative to the time course of the reaction.

In the case of both dialyzed and undialyzed plasma, very slight activation was obtained at concentrations between 0.001 and 0.002 M in the reaction mixture as shown in Charts XIX and XX. Concentrations as high as the 0.00625 M used by Bodansky (13) (14) definitely produced inhibition. After dialysis, the activation produced by the addition of glycine was very small and its role under the conditions of these experiments may be insignificant.

The effect of starving of the chicks to deplete their blood of amino acids was also studied in relation to the glycine activation

Chart XVII

Effect of Magnesium and Manganese on the Phosphatase Activity of Dialyzed and Undialyzed Chick Plasma.

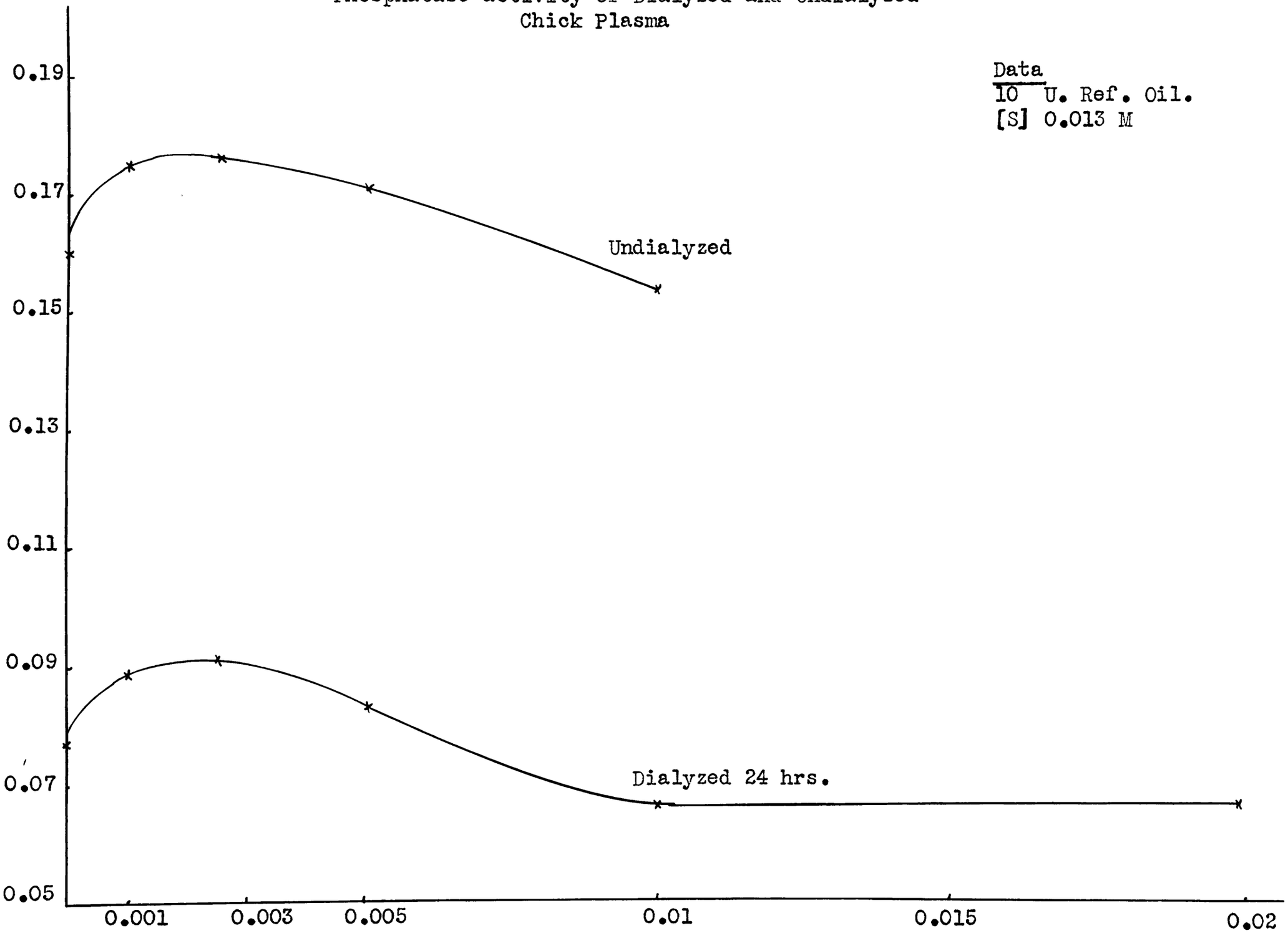


M Concentration of Magnesium in digest.
M Concentration x 10 of Manganese in digest.

Chart XVIII

Effect of Low Concentrations of Manganese ion on the Phosphatase activity of Dialyzed and Undialyzed Chick Plasma

Data
10 U. Ref. Oil.
[S] 0.013 M



Effect of Glycine on the Activity of the Phosphatase of Chick Plasma

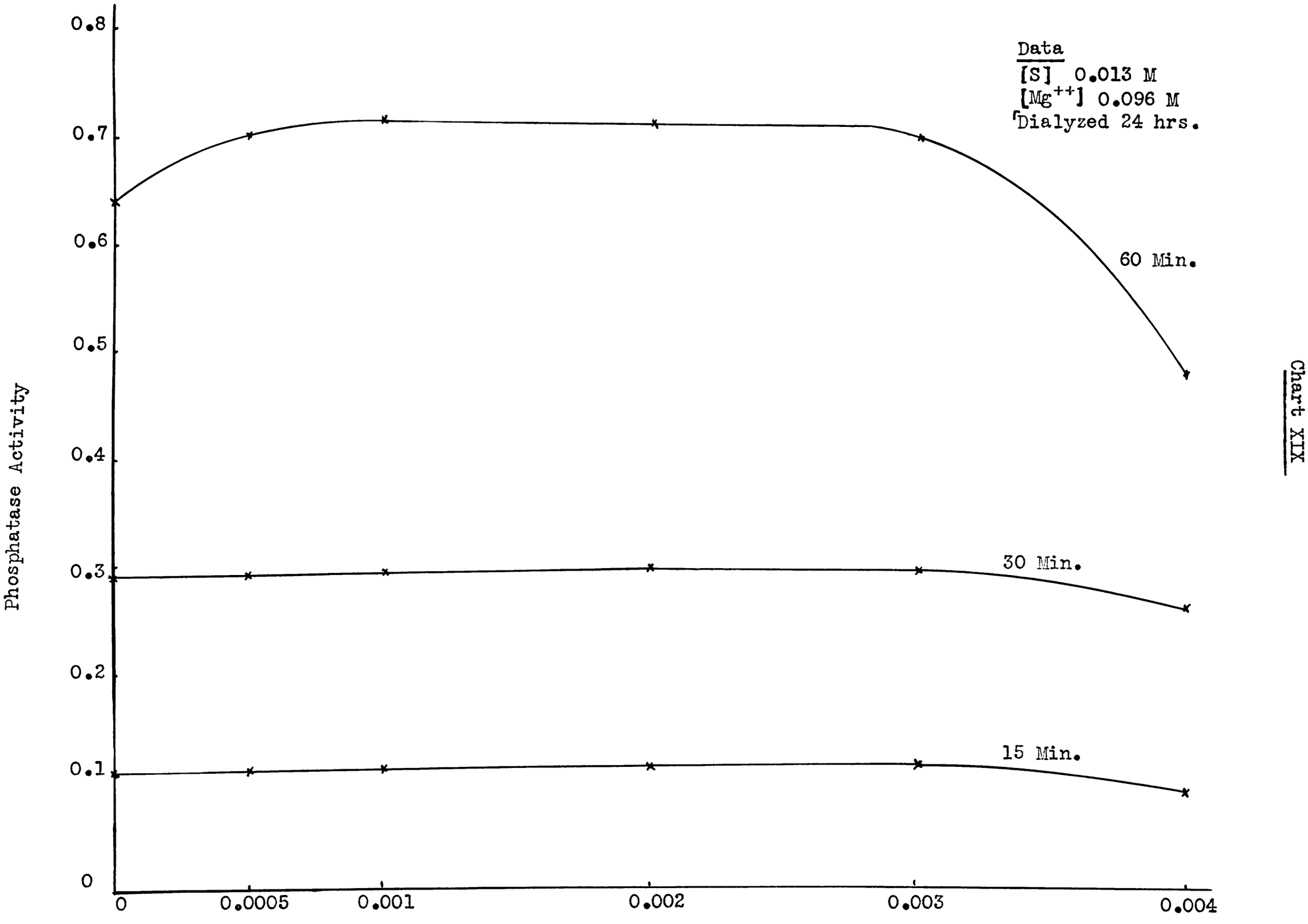


Chart XIX

Effect of Glycine on the Activity of the Phosphatase of Chick Plasma.

Data Jan. 10/41
10 I. U. D. Ref. Oil
[S] 0.025 M
[Mg^{++}] 0.009 M

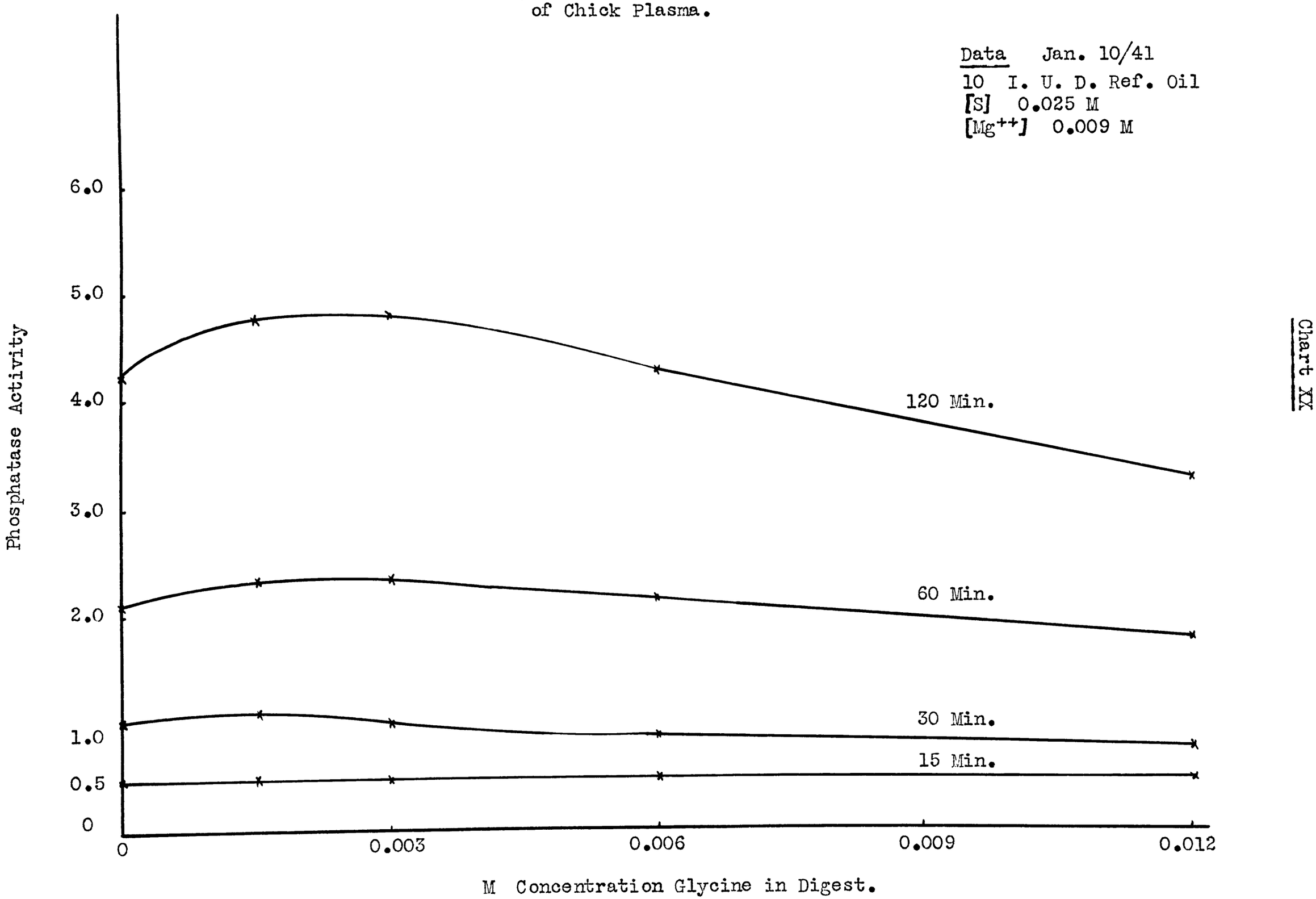


Chart XX

problem. Blood samples were taken from nine three-week-old chicks by the heart stab method. These birds were starved for 18 hours and the second set of samples of blood was taken, this one by the severing of the neck. No glycine was added in these experiments and the results presented in Table VIII show that there was a slight decrease in phosphatase activity after starvation. A similar decrease in activity of the plasma phosphatase of starved adult fowl has been reported by Auchinachie and Emslie (3). The decreased activity observed in the present experiments may be attributed to the decrease in α-amino acids in the blood or it may be due to an upset in the physiological order of the starved chick. Because the amount of blood which could be obtained from individual chicks was insufficient for further study, no comparative tests were made adding glycine.

In another experiment, nine chicks were sampled without starving and nine pen mates were sampled after starving 18 hours. The results of these tests are shown in Table IX, and it may be observed that the phosphatase activities of the plasmas of starved and unstarved chicks were practically identical at the various concentrations of glycine.

The effect of varying concentrations of glycine on the time course of hydrolysis is shown in Charts XXI and XXII. These data would also suggest the presence of an induction period similar to that found in Chart III in the study of initial velocities. This induction period has since been removed by reducing the concentration of ammonium ion and increasing the concentration of magnesium ion.

(cf. time of hydrolysis, page 16)

Table VIII

The Effect of Starvation of Chicks on the Activity of the Plasma Phosphatase.

Blood samples removed from nine individual chicks by the heart stab method. Chicks starved 18 hours and resampled.

Chick No.	Weight gms.	Bone Ash %	Activity (fed) (mgms.)	Activity (starved) (mgms.)
4363	161	44.4	0.221	0.193
4364	182	44.7	0.184	0.140
4366	135	47.5	0.114	0.089
4369	170	47.3	0.141	0.116
4371	95	44.9	0.242	0.169
4372	149	45.0	0.222	0.123
4381	155	----	0.221	0.176
4387	173	46.3	0.346	0.279
4388	165	44.2	0.208	0.173
Average			0.211	0.162

Data - Temp. 30°C.

Time of Digestion - 15 minutes.

Magnesium ion conc. in digest - 0.009 M.

Substrate " " " - 0.025 M.

No added glycine.

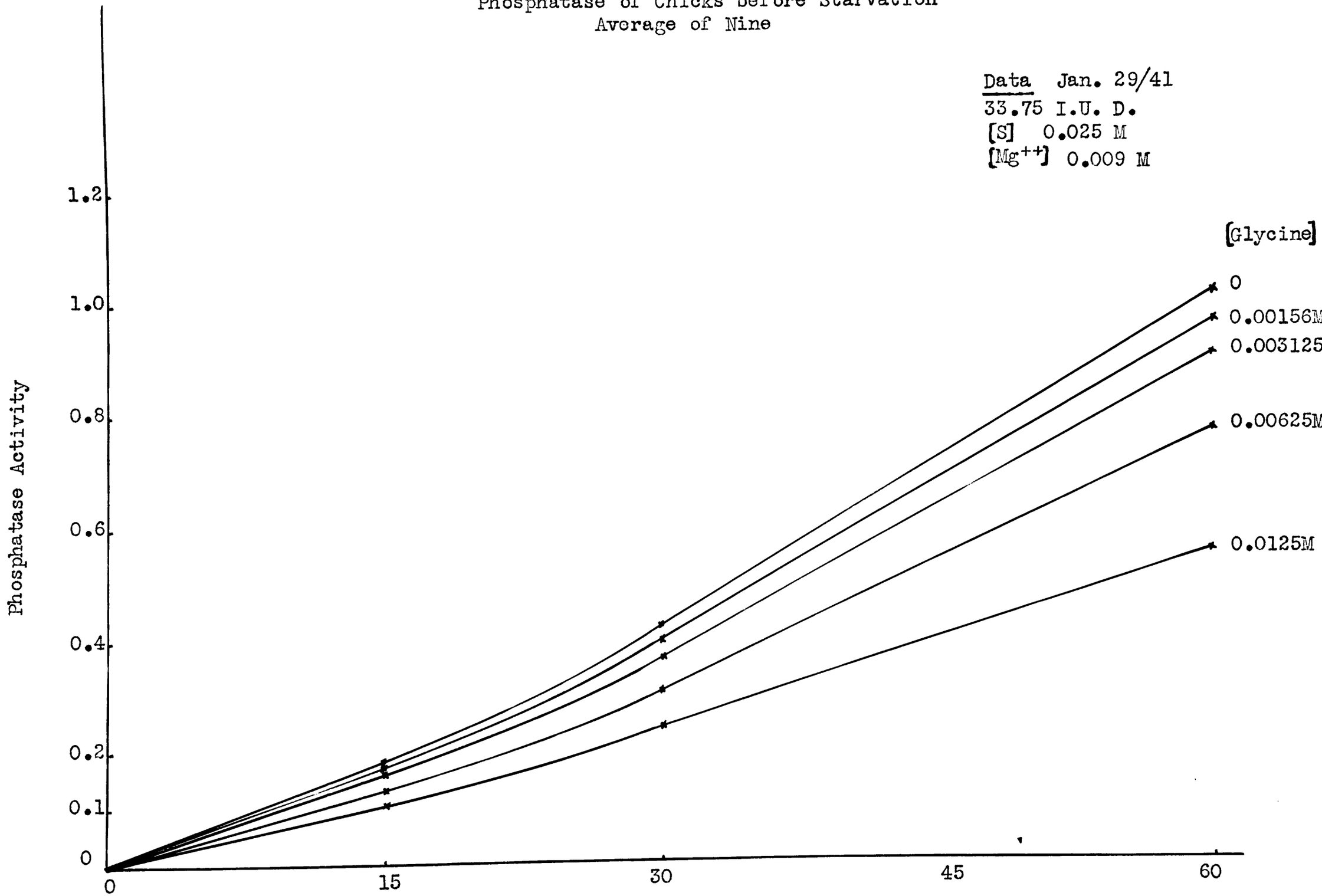
Table IX.

Effect of Added Glycine on the Phosphatase Activity of the Blood of Starved and Unstarved Chicks.

Glycine Concentration	Activity	
	Unstarved	Starved
0.0	0.184	0.174
.00156	0.167	0.176
.00313	0.162	0.165
.00625	0.136	0.141
.01250	0.117	0.123

Effect of Glycine on the Activity of the Plasma Phosphatase of Chicks before Starvation
Average of Nine

Data Jan. 29/41
33.75 I.U. D.
[S] 0.025 M
$[Mg^{++}]$ 0.009 M



Effect of Glycine on the Activity of the Plasma Phosphatase of Chicks after 18 hrs. Starvation. Average of Nine

Data Jan 30/41
33.75 I. U. D/100 gm feed
[S] 0.025 M
$[Mg^{++}]$ 0.009 M

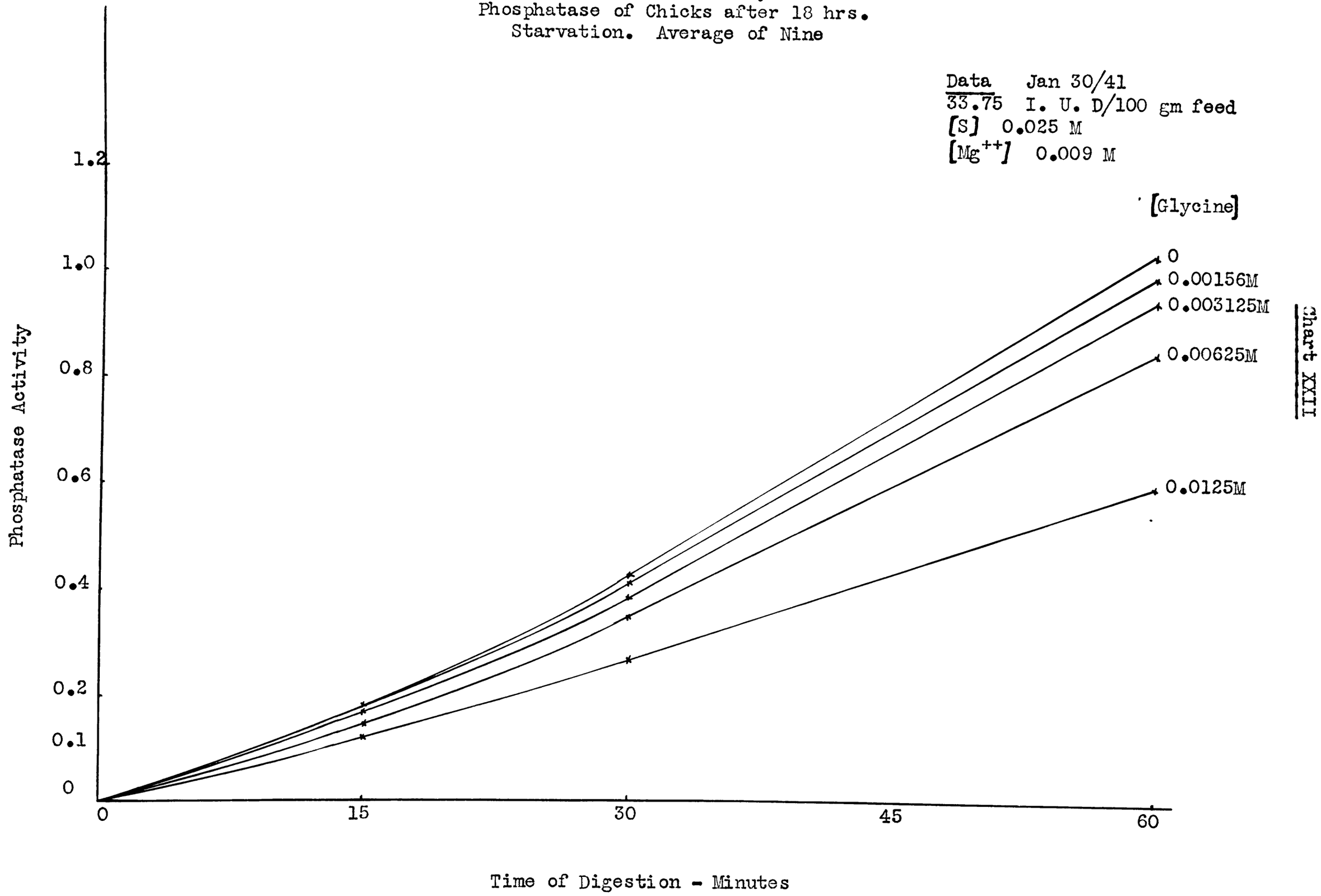


Chart XXII

Oxalate Inhibition.

It has been claimed by Munemura (62), that oxalate completely inhibits the alkaline phosphatase in extracts of hog kidney. Belfanti, Contardi and Ercoli (10) disagree with this, finding practically no inhibition of alkaline phosphatase in extracts of rabbit liver and kidney. However, the same workers report considerable inhibition of the alkaline phosphatase of horse and rabbit sera by oxalate. Bodansky (11) reports that the average activity ratio of oxalated plasma phosphatase is 0.9, when the activity of the serum enzyme is taken as 1.0. Auchinachie and Emslie (3) could find no significant difference between oxalated blood plasma and serum in chicks.

In the present work no difference could be found between the phosphatase activity of oxalated blood plasma solution and plasma obtained by rapidly adding a known amount of fresh blood to saline solution and centrifuging. The data presented in Table X suggested that the oxalate concentration in the reaction mixture under the conditions of this experiment (about 0.005 per cent) was too small to have an appreciable effect.

Hemolysis.

Bodansky (11) reports slight hemolysis to have no appreciable effect on the phosphatase activity in serum phosphatase determinations.

In the present work, no samples have been encountered where more than slight hemolysis occurred. Complete hemolysis, brought about by the use of distilled water in place of the 0.9 per cent sodium chloride solution used in diluting the blood, reduced the phosphatase activity by about one third as shown in Table XI.

Table X

The Effect of Oxalate on the Activity of Plasma Phosphatase of Chicks.

Chick No.	Activity	
	Oxalated	Not Oxalated
6054	0.165	0.154
6056	0.655	0.577
6042	0.388	0.399
6043	0.423	0.423
Average	0.408	0.388

Table XI

The Effect of Hemolysis on Phosphatase Activity

Treatment	Activity		
	Sample No. 1	Sample No. 2	Sample No. 3
Unhemolyzed	0.122	0.254	0.404
Hemolyzed	0.091	0.152	0.272

METHOD

Small glass vials are prepared for receiving the blood samples by adding 0.2 cc. of 2.5% sodium oxalate to each and drying them. Individual blood samples from each chick are collected in these vials, (about 2.5 cc. is sufficient). The blood is obtained by skinning the neck, separating the gullet and veins away and cutting the neck with a pair of scissors. The chicks are not previously starved.

0.5 cc. of blood from each chick are pooled and 1 cc. of the pooled sample added to 20 cc. of 0.9% sodium chloride solution in a centrifuge tube and centrifuged at about 2000 r.p.m. for 10 minutes. The phosphatase activity of this preparation kept at 4°C. in a glass-stoppered bottle, is determined within 2 hours.

Digest

The reaction mixture consists of 1 part sodium β-glycerophosphate substrate solution, 1 part buffer, and 2 parts blood plasma saline solution. The substrate and buffer are mixed and placed in a flask in the water-bath for 30 minutes to come to temperature. The blood plasma solution is similarly brought to temperature, suitable aliquots (5 cc.) being pipetted into thick-walled hard-glass stoppered test-tubes and placed in the water-bath. At the proper time, 5 cc. of the substrate-buffer solution is added to the 5 cc. of plasma solution and, after replacing the stopper, the tube is mixed by inversion.

Immediately after the two solutions have been mixed, a 4 cc. aliquot is removed and added to 3 cc. of 15% trichloracetic acid in a centrifuge tube for a zero-time blank. The remainder is corked and allowed to stand in the water-bath at 30°C for 15 minutes. Then the tube is shaken

and another 4 cc. aliquot is removed, added to 3 cc. of 15% trichloracetic and centrifuged. 2000 r.p.m. for 5 minutes is sufficient to throw down all precipitated protein.

Phosphorus is determined as follows:-

A 4 cc. aliquot of the solution to be analyzed is placed in a 10 cc. volumetric flask. To this is added 1.0 cc. of ammonium molybdate-sulphuric acid reagent and 0.5 cc. of amino naphthol sulphonic acid reagent. The solutions are made up to volume, shaken thoroughly and the intensity of colour determined in the photelometer 30 minutes after the addition of the reducing agent.

Results are expressed as milligrams of P liberated in 15 minutes by the phosphatase in 1 cc. of blood.

Reagents.

1. 0.9% sodium chloride solution

   9 gms. 'Analar' Sodium chloride/litre.

2. Buffer

   165 cc. 1 M Ammonium hydroxide C.P.
   35 cc. 1 M Ammonium Chloride, B.D.H. cert.) / litre.

   made up to volume with CO_2 free distilled water.

3. Substrate solution

   800 cc. 0.065 M sodium β-glycerophosphate, B.D.H.
   200 cc. 1.0 M magnesium chloride, B.D.H. cert.

4. Trichloracetic acid (15%).

   150 gms. trichloracetic, 'Analar' or B.D.H. cert.

5. Ammonium molybdate-sulphuric acid reagent.

2½% Ammonium molybdate solution, B.D.H. cert.
10 N Sulphuric acid, C.P.
(279 cc. conc. H_2SO_4/l)

Mix in proportion

100 cc. molybdate solution
45 cc. sulphuric acid
105 cc. distilled water

Made up directly before using.

6. Amino Naphthol sulphonic acid reagent.

0.2 gms. 1,2,4 amino naphthol sulphonic acid B.D.H.(extra pure)
12.0 gms. sodium bisulphite Baker's gran.
2.4 gms. sodium sulphite, B.D.H. cert.

Dissolve and make up to 100 cc. with distilled water. Filter if necessary. Keep cold and dark. Never keep longer than 2 weeks.

7. 2.5% $Na_2C_2O_4$, 'Analar'.

OTHER INVESTIGATIONS

Variability of Chicks.

One of the most likely places to encounter error in the proposed chick phosphatase assay for vitamin D carriers would be in the tremendous individual variation among chicks on the same diet. In an attempt to discover if this variation were due to inherited characteristics only, some pedigreed chicks were put on experiment. The treatment given was the same as that for ordinary assays.

Group No. 1 comprised pen pedigreed chicks only. That is, the chicks were known to be the progeny of one dam and one or more sires, and the chicks from any one dam would therefore be at least half-brothers and sisters. Group No. II was composed of the progeny of single matings. In either case, all chicks from one dam were fed on the same ration.

Phosphatase activity and bone ash determinations were run on each chick. The results for analyses on Group No. 1 are found in Table XII, and those for Group No. II are found in Table XIII.

It will be noted that brother and sister chicks in either group, as a rule, bore no marked similarity in either phosphatase analyses or in bone ash determinations, although, in the bone ash, the variation from chick to chick in the progeny of any one dam was less than the variation among the progeny of different dams. The number of chicks from each hen is hardly sufficient to draw any conclusion concerning the variability of phosphatase activity.

These findings would tend to corroborate in part the report of O'Neil(66), although his work was done on day-old chicks and comparison may not be justified.

Table XII

Phosphatase Activity of Pen Pedigreed Chicks

Hen No.	Chick No.	Weight gms.	Sex	Units D per 100 gm. feed	Bone Ash%	Phosphatase Activity	Aver.
1	H6961	118	F	0	30.4	0.970	
	H6962	170	M	0	27.0	0.886	
	H6963	156	F	0	28.5	1.160	
	H6964	140	F	0	29.8	0.631	0.912
2	H6965	129	F	0	28.2	1.457	
	H6966	134	F	0	30.4	1.032	
	H6967	142	F	0	31.1	0.944	
	H6969	136	F	0	32.0	0.881	
	H6978	150	M	0	30.7	0.750	1.013
3	H6973	129	F	0	26.6	1.110	
	H6974	140	M	0	29.7	0.694	
	H6975	106	F	0	29.0	1.457	
	H6976	148	M	0	27.5	1.256	
	H6977	142	M	0	29.9	1.310	1.165
4	H7013	146	M	10	31.0	0.881	
	H7014	173	M	10	30.5	0.714	
	H7015	147	F	10	30.3	1.205	
	H7016	168	M	10	----	0.792	
	H7017	173	M	10	28.3	1.000	0.918

Table XII (cont'd)

Hen No.	Chick No.	Weight gms.	Sex	Units D per 100 gm. feed	Bone Ash %	Phosphatase Activity	Aver.
5	H7026	110	F	10	33.7	0.970	
	H7027	141	M	10	31.1	0.771	
	H7028	147	M	10	29.9	0.880	
	H7029	135	M	10	30.8	0.857	
	H7030	155	M	10	30.7	0.756	
	H7031	143	M	10	30.7	0.771	
	H7032	155		10	29.5	0.787	0.824
6	H7042	205	F	15	37.6	0.406	
	H7043	168	F	15	40.1	0.312	
	H7044	168	F	15	----	0.184	
	H7045	160	F	15	33.0	0.614	
	H7046	206	F	15	34.6	0.544	
	H7047	147	F	15	33.9	0.814	
	H7048	190	M	15	33.0	0.712	
	H7049	212	M	15	37.9	0.470	0.507
7	H7059	280	M	22.5	37.5	0.347	
	H7060	180	F	22.5	35.0	0.495	
	H7061	194	F	22.5	38.8	0.860	
	H7062	222	F	22.5	39.6	0.517	
	H7063	173	F	22.5	34.1	0.661	
	H7064	213	F	22.5	39.5	0.495	0.562

Table XIII

Phosphatase Activity of Pedigreed Chicks (Individual Matings).

Hen No.	Chick No.	Weight gms.	Sex	Units D per 100 gm. feed	Bone Ash %	Phosphatase Activity	Aver.
3140	11143	152	M	10	36.1	0.437	
	11144	143	M	10	37.4	0.694	
	11145	113	F	10	33.3	0.483	
	11146	167	F	10	34.4	0.382	0.499
3204	11147	130	M	10	30.2	0.876	
	11148	135	M	10	32.1	0.902	
	11149	82	F	10	32.1	0.835	
	11150	118	F	10	32.7	0.617	
	11151	120	F	10	32.5	0.789	
	11152	104	F	10	26.8	0.601	
	11153	129	M	10	29.5	0.646	0.752
3263	11154	155	M	10	34.0	0.499	
	11155	158	F	10	30.2	0.611	
	11156	172	M	10	33.9	0.631	0.580
3250	11157	95	M	10	29.4	0.711	
	11160	91	M	10	26.0	0.447	0.579
3259	11161	149	M	10	28.0	0.766	0.766
3212	11162	127	F	10	31.5	0.476	
	11163	125	F	10	29.2	0.440	
	11164	137	M	10	30.3	0.788	
	11165	127	F	10	27.6	0.457	
	11166	127	F	10	30.6	0.638	0.560

Table XIII (cont'd)

Hen No.	Chick No.	Weight gms.	Sex	Units D per 100 gm. feed	Bone Ash %	Phosphatase Activity	Aver.
3019	11188	116	F	10	31.5	0.600	
	11189	107	F	10	30.4	0.437	
	11190	124	M	10	30.8	0.501	
	11191	130	M	10	32.1	0.466	
	11192	136	M	10	32.8	0.416	
	11193	129	F	10	31.7	0.284	0.451
3214	11168	154	M	33.75	40.8	0.165	
	11169	123	F	33.75	37.4	0.268	
	11170	173	M	33.75	41.8	0.259	
	11171	153	M	33.75	43.1	0.139	
	11172	113	M	33.75	38.4	0.248	0.219
3207	11173	147	M	33.75	47.4	0.119	
	11174	131	M	33.75	45.2	0.101	
	11175	175	F	33.75	43.9	0.150	
	11176	130	M	33.75	42.4	0.205	0.144
3247	11178	170	M	33.75	44.2	0.308	
	11179	184	M	33.75	44.1	0.132	
	11180	169	F	33.75	42.2	0.106	0.182
3241	11181	163	M	33.75	42.0	0.134	
	11182	173	F	33.75	41.3	0.161	
	11183	164	F	33.75	35.5	0.270	
	11185	110	F	33.75	44.0	-----	0.188

Variability of Ducks.

Considering the results from the pedigreed chicks where the individual variation in bone ash per cent and in phosphatase activity was still high among the progeny of a single mating, it was decided to try a very homogeneous strain of ducks which were available. These ducks had been inbred for over thirty years and were therefore of a genetically homogeneous nature.

Before tests on individuals were made, it was thought advisable to establish the optima for the duck blood plasma phosphatase. The optimum pH was found to be around 9.7 as it was for the chicks. The magnesium ion optimum concentration was about 1/10 of the optimum concentration for chicks, being 0.005 M in the digest.

As was expected, the variation among individuals on the same diet was very much smaller than that found among chicks under similar conditions. The results are to be found in Table XIV.

Table XIV

Plasma Phosphatase of Ducks.

Units D per 100 gm. feed	Duck No.	Weight	Bone Ash %	Phosphatase Activity	Aver.
0	11203	295	37.0	0.328	
	11205	200	35.0	0.257	
	11206	245	36.6	0.270	
	11209	150	36.3	0.279	
	11212	175	34.2	0.244	
	11213	200	38.0	0.200	
	11216	155	38.6	0.220	0.257
15	11256	275	38.3	0.163	
	11260	312	38.2	0.191	
	11263	158	38.3	0.158	
	11264	350	39.3	0.198	
	11266	430	39.1	0.211	
	11268	386	36.9	0.279	
	11270	270	35.8	0.270	0.210
Pooled Samples					
4.44			33.9	0.281	
10.			36.4	0.272	
22.5			40.3	0.204	
33.75			43.4	0.119	

Correlation Between Phosphatase Activity and Dosage of Vitamin D.

An extensive experiment was undertaken to determine, if possible, at what age of the chick the relationship between phosphatase activity and dosage of vitamin D, or between phosphatase activity and bone ash percentage, proved the closest.

In this experiment, eight pens of twenty chicks each were put on each of four rations containing 0 I. Units D, 10 I. Units of D, 22.5 I. Units D, and 41.5 I.Units D per 100 grams of ration respectively. The source of the vitamin D was National Oil Products Nopco XX 400 D Fortified Cod Liver Oil. The regular A.O.A.C. chick assay (62) on this oil showed a potency of 460 I. Units of D per gram. Of these chicks, one pen from each ration was killed each week for eight weeks. From pooled samples of blood from each pen, the phosphatase activity of the plasma was determined on three levels of substrate. Bone ash was done on the left tibia of each individual chick according to the A.O.A.C. method prescribed for vitamin D assay. Results are tabulated in Table XV.

From a consideration of the data in Chart XXIII, it will be seen that the degree of relationship between phosphatase activity and the dose approaches its maximum when the chicks are four or five weeks old.

From Chart XXIV, it may be seen that the greatest degree of correlation between bone ash per cent and phosphatase activity is to be found at four or five weeks.

Statistical analyses of results obtained from determinations of the phosphatase activity of individual chicks as compared with their bone ash per cent showed a high degree of correlation. The value of the coefficient

obtained was r=0.69 where n equalled 44. Since the required coefficient for odds of P=.01 is 0.354 this would indicate that the relationship is a real one.

Table XV

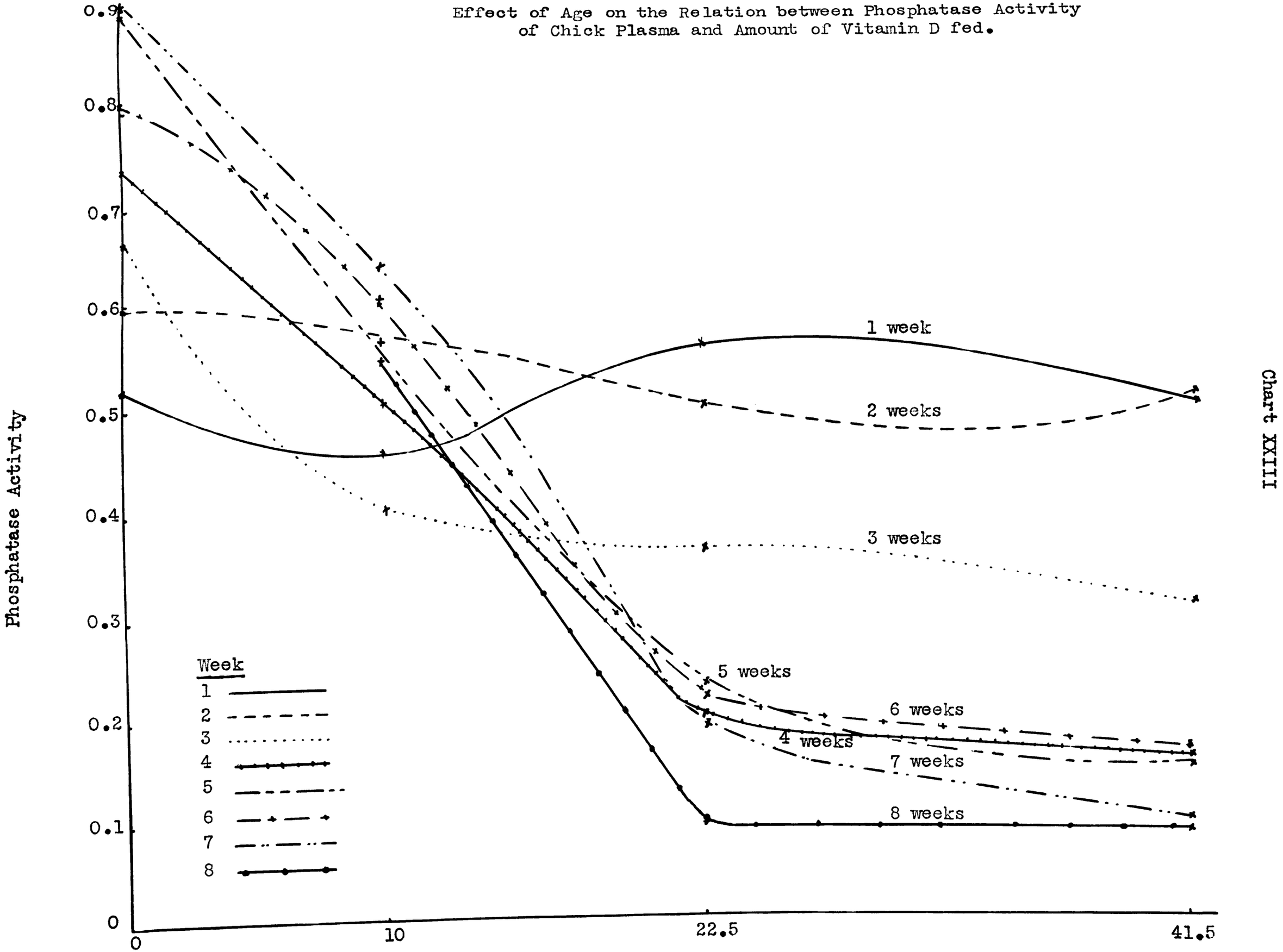
Phosphatase Activity on Four Levels of Vitamin D at Weekly Intervals.
Km and Vmax

Week	Units D per 100 gms. of ration.	No. of Survivors	Bone Ash %	Activity at 0.013 M Subst.	Vmax.	Km.
1	0	19	35.94	0.518	1.830	0.0353
	10	20	36.55	0.461	1.100	0.0213
	22.5	19	39.00	0.563	1.730	0.0282
	41.5	19	40.46	0.512	1.670	0.0305
2	0	19	32.31	0.608	1.560	0.0213
	10	19	33.85	0.575	1.345	0.0190
	22.5	20	42.16	0.504	1.345	0.0235
	41.5	19	42.44	0.518	1.804	0.0328
3	0	20	31.06	0.675	1.427	0.0140
	10	19	34.69	0.407	0.783	0.0123
	22.5	20	39.53	0.362	0.836	0.0171
	41.5	18	44.88	0.310	0.747	0.0189
4	0	20	30.52	0.739	1.312	0.0101
	10	23	34.30	0.509	0.872	0.0096
	22.5	19	41.02	0.200	0.430	0.0141
	41.5	19	44.42	0.164	0.216	0.0090
5	0	16	30.97	0.902	1.359	0.0063
	10	21	36.33	0.569	0.775	0.0048
	22.5	19	42.87	0.246	0.320	0.0043
	41.5	19	45.79	0.154	0.221	0.0049
6	0	11	31.70	0.807	1.110	0.0049
	10	21	36.44	0.609	0.813	0.0044
	22.5	19	42.20	0.218	0.288	0.0044
	41.5	14	44.63	0.173	0.231	0.0044
7	0	7	33.00	0.918	1.218	0.0036
	10	17	34.90	0.645	0.928	0.0054
	22.5	16	45.38	0.189	0.319	0.0086
	41.5	19	43.60	0.099	0.157	0.0075
8	0	--	--	--	--	--
	10	17	37.13	0.554	0.827	0.0067
	22.5	16	43.66	0.095	0.137	0.0060
	41.5	17	44.29	0.086	0.156	0.0103

Vmax and Km calculated by the equations of Michaelis and Menten (60).

Chart XXIII

Effect of Age on the Relation between Phosphatase Activity of Chick Plasma and Amount of Vitamin D fed.



Effect of Age on Relation Between Bone Ash and Plasma Phosphatase Activity of Chicks.

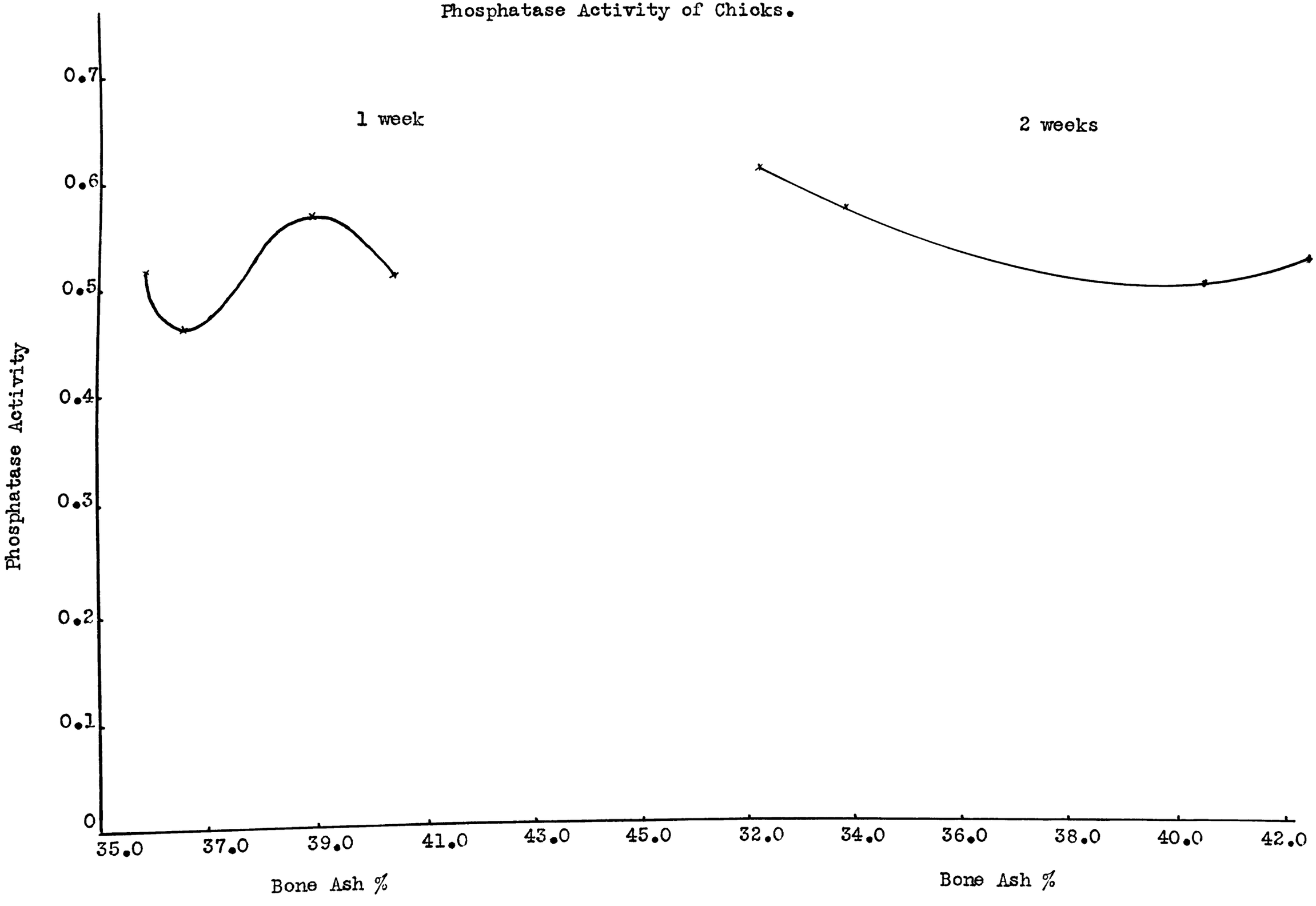


Chart XXIV

Bone Ash vs Phosphatase Activity

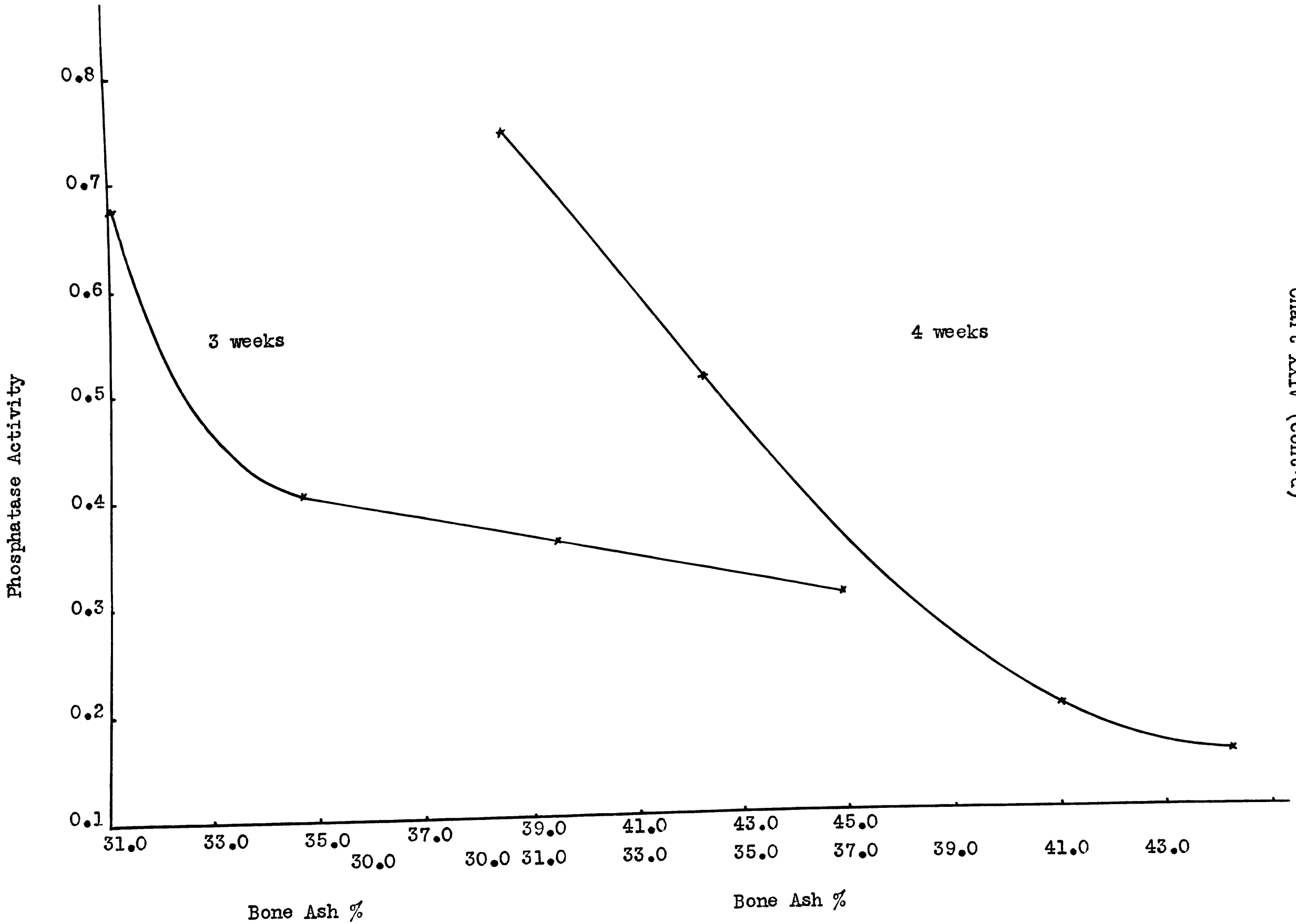


Chart XXIV (cont'd)

Chart XXIV (cont'd)

Bone Ash vs Phosphatase Activity

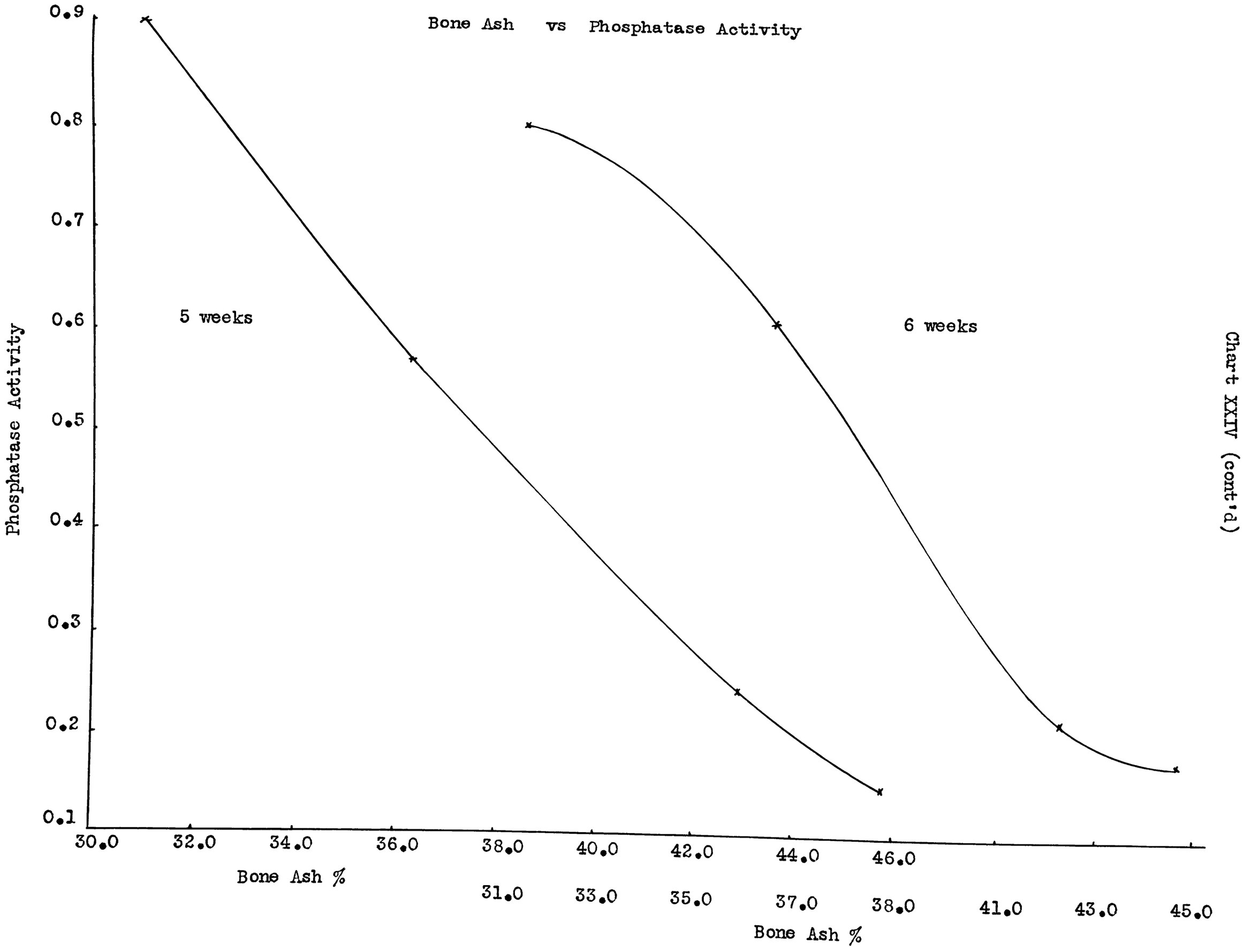
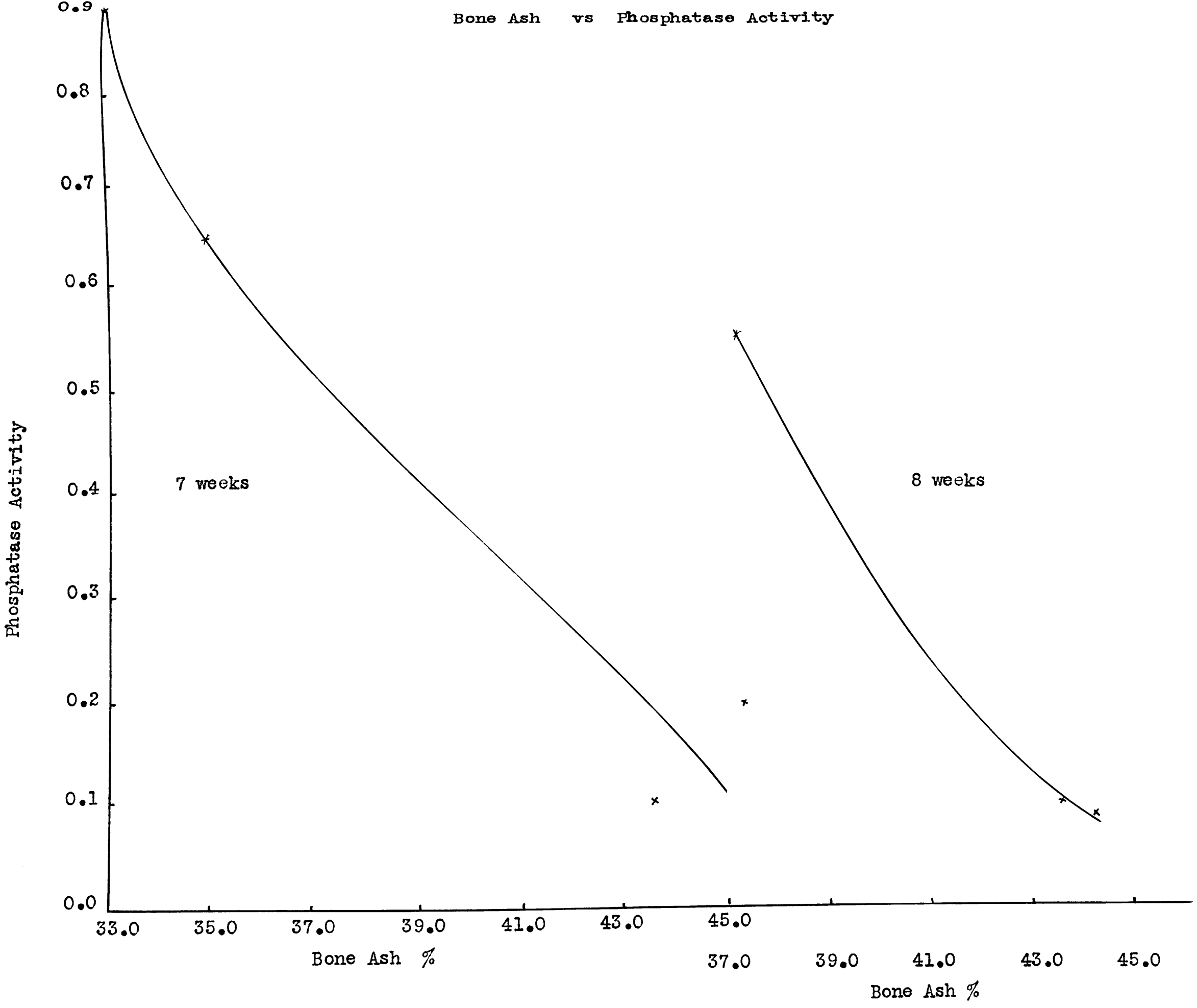


Chart XXLV (Cont'd)

Bone Ash vs Phosphatase Activity



Enzyme Kinetics.

The Km's for the phosphatase activity of pooled samples of blood from the pens of chicks on varying doses of vitamin D and at various ages showed a decrease as time went on, reaching a minimum and levelling off for all doses at six weeks. Admittedly an insufficient number of levels of substrate were used but when these data were plotted out and more points taken for the algebraic calculations, according to the equation of Michaelis and Menton (61), the results obtained were very similar. These data are shown in Table XVI.

Km's calculated by the equation of Lineweaver and Burk (54) as compared with those calculated by the Michaelis-Menten equation are shown in Table XVII.

The Km's obtained on individual chicks earlier in the work, using 0.009 M concentration of magnesium ion in the digest, proved to be very low as compared with those obtained on pooled samples using 0.05 M magnesium ion and 0.002 M glycine. Some of these early Km's are presented in Tables XVIII and XIX.

With the idea that perhaps the low concentration of magnesium ion used in the earlier work was responsible for the low Km's, substrate-activity curves were run, using two levels of magnesium ion concentration on the same plasma solution. Using 0.05 M magnesium ion, the Km was 0.00714 and the Vmax. 0.493. Using 0.0091 M magnesium ion, the Km was 0.0056 and the Vmax. 0.463. This difference was not great enough to explain the earlier discrepancy.

Table XVI

Comparison of Km and Vmax calculated algebraically using varying numbers of substrate levels.

Original Data.

Subst. Conc.	Activity
0.003	0.137
0.006	0.154
0.009	0.171
0.012	0.173
0.018	0.173

Km - 0.0014
Vmax - 0.194

A curve was plotted from this data and the following information taken from the graph:

Subst. Conc.	Activity
0.001	0.068
0.002	0.112
0.003	0.135
0.004	0.147
0.005	0.155
0.006	0.161
0.007	0.167
0.008	0.170
0.009	0.171
0.010	0.172

Calculating Vmax and Km from these ten points -

Vmax - 0.2113 Km - 0.0019

Using the first six points, i.e. 0.001 - 0.006 inclusive

Vmax - 0.227 Km - 0.0022

Using the middle six points, i.e. 0.003 - 0.008 inclusive

Vmax - 0.202 Km - 0.0015

Using the last six points, i.e. 0.005 - 0.010 inclusive

Vmax - 0.202 Km - 0.0016

Table XVII

Km and Vmax calculated by the equation of Michaelis and Menten (61) and by the equation of Lineweaver and Burk (54)

Michaelis-Menten Algebraic Method		Lineweaver-Burk Algebraic Method		Units D	Week
Vmax.	Km.	Vmax.	Km.		
1.830	0.0353	1.800	0.0328	0.0	1
1.100	0.0213	1.485	0.0292	10.0	
1.730	0.0282	1.955	0.0318	22.5	
1.670	0.0305	1.490	0.0247	41.5	
1.560	0.0213	1.510	0.0192	0.0	2
1.345	0.0190	1.370	0.0180	10.0	
1.345	0.0235	1.335	0.0216	22.5	
1.804	0.0328	1.835	0.0334	41.5	
1.427	0.0140	1.370	0.0134	0.0	3
0.782	0.0123	0.690	0.0094	10.0	
0.836	0.0171	0.817	0.0165	22.5	
0.747	0.0189	0.788	0.0204	41.5	

Table XVIII

Chick	Sex	Weight	Bone Ash %	[M] Na β-Glycerophosphate (.003)	(.006)	(.009)	(.012)	(.018)	Vmax.	Km.
				Phosphatase Activity						
4453	F	154	41.3	0.158	0.191	0.217	0.219	0.228	0.253	.0018
4454	M	167	39.2	0.256	0.311	0.342	0.348	0.355	0.389	.0014
4455	F	164	45.7	0.137	0.154	0.171	0.173	0.173	0.194	.0014
4456	F	149	44.0	0.138	0.171	0.180	0.184	0.188	0.204	.0013
4457	M	138	34.3	0.307	0.379	0.395	0.432	0.434	0.478	.0017
4458	M	157	39.9	0.324	0.384	0.417	0.428	0.422	0.484	.0015
4466	F	133	36.0	0.235	0.285	0.312	0.324	0.338	0.361	.0016
4467	M	208	45.8	0.160	0.176	0.208	0.188	0.188	0.239	.00165
4468	F	114	41.1	0.235	0.281	0.307	0.307	0.294	0.362	.00167
4463	F	181	44.2	0.217	0.239	0.248	0.244	0.239	0.268	.0007
4464	F	155	42.6	0.151	0.163	0.171	0.176	0.176	0.185	.00073
4465	F	140	42.4	0.136	0.176	0.184	0.189	0.197	0.216	.0016

Data - 22.5 I. U. Ref. Oil

15 min. at 30°C.

.009 M $MgCl_2$

No added glycine.

Table XIX

Chick	Sex	Weight	Bone Ash %	[M] Na β-Glycerophosphate (.003)	(.006)	(.009)	(.012)	(.018)	Vmax.	Km.
				Phosphatase Activity						
4413	F	103	35.2	0.314	0.395	0.435	0.463	0.485	0.545	.0022
4419	F	107	44.8	0.150	0.161	0.165	0.158	0.151	0.176	.00056
4426	F	82	31.8	0.313	0.406	0.435	0.454	0.463	0.520	.0019
4421	F	140	34.4	0.280	0.375	0.405	0.415	0.445	0.502	.0023
4427	M	153	33.8	0.256	0.309	0.331	0.340	0.355	0.385	.0015
4429	F	129	31.3	0.332	0.428	0.500	0.516	0.548	0.629	.0018
4411	M	108	37.1	0.358	0.510	0.554	0.570	0.552	0.755	.0030
4424	M	170	36.0	0.276	0.392	0.441	0.441	0.443	0.520	.0023
4423	M	145	34.0	0.217	0.278	0.309	0.325	0.327	0.427	.0032
4415	F	89	30.8	0.184	0.251	0.256	0.266	0.266	0.316	.0020
4416	F	119	36.2	0.646	0.879	1.000	1.045	1.095	1.290	.0029
4417	M	119	39.9	0.237	0.285	0.306	0.320	0.334	0.363	.0024

Data - 10 I. U. Ref. Oil

15 min. at 30°C.

.009 M $MgCl_2$

no added glycine.

VITAMIN D BIOASSAYS PERFORMED

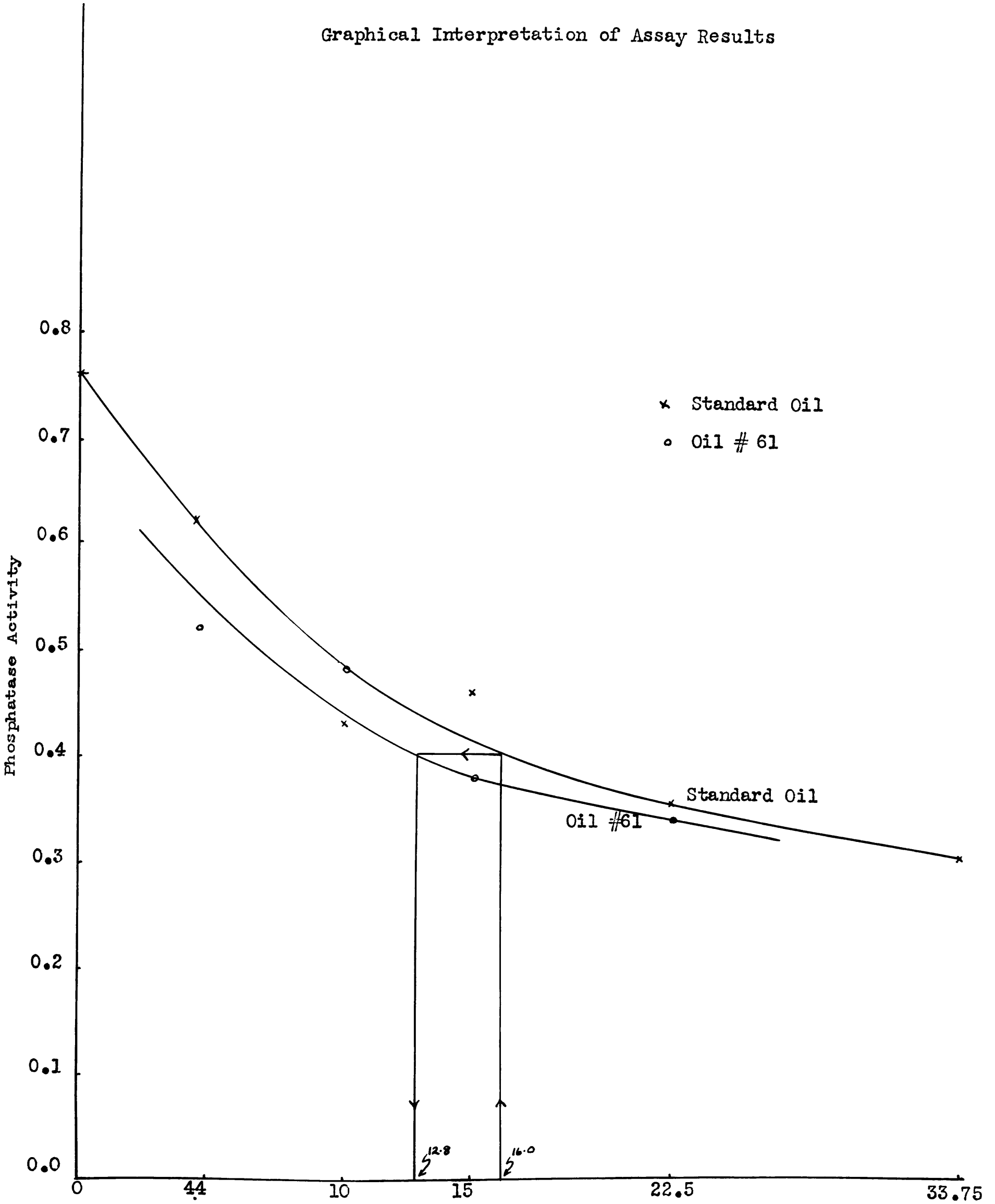
Vitamin D bioassays, using the phosphatase activity of the chick blood plasma as well as the regular assay by the bone ash method, were performed on eight vitamin D carriers. Three of these preparations were fish oils, two were synthetic vitamin D_3, and three were dry preparations. These carriers were fed, assuming the quoted potencies as correct, in the regular chick bioassay as prescribed by the A.O.A.C. method. It is unfortunate that this particular part of the work was incidental to the regular vitamin D chick bioassays and the phosphatase analyses were necessarily carried out when the chicks were three weeks of age. Perhaps better results might have been obtained if the analyses had been done when the chicks were four weeks old.

At the end of the twenty-one day feeding period, blood samples were collected from each chick in a pen, pooled samples prepared and phosphatase activity determinations carried out as previously described. Curves of the activity, as plotted against the vitamin D fed, were obtained, as shown in Chart XXV, and the potency of the carrier under assay determined by comparison with the curve of response of the chicks on Canadian Reference Oil. Drawing a curve of best fit is necessarily a matter of controversy and here it seems apparent that larger groups of chicks and a longer feeding period would smooth out the points to a considerable extent.

Example. - By reading up from 16 on the I. Unit base line, in Chart XXV, to the standard oil curve, across to the curve for oil # 61, and back down to the base line, the value 12.8 is obtained. This value, when divided into 16 gives a factor of 1.25. Similar factors are derived

Chart XXV

Graphical Interpretation of Assay Results



at various points along the curve and the results averaged. The average value, multiplied by the assumed potency of the oil, gives the biological assay result. These calculations for oil # 61 are shown in Table XX.

Table XX.

Calculations in Determining the Potency of Oil # 61

I. Unit base line	1st reading	2nd reading	Factor.
	24	20.0	1.20
	22	18.0	1.22
	20	16.0	1.25
	18	14.3	1.26
	16	12.8	1.25
	14	11.2	1.25
	12	9.5	1.27
	10	7.6	1.32
		8	10.02
		Average -	1.25

Assumed potency of oil = 400 I. U. vitamin D per gram of oil.

Assayed potency of oil = 400 x 1.25 = 500 I.U. vitamin D per gram of oil.

The phosphatase activity values for the pens of chicks on the vitamin D carriers assayed, are contained in Table XXI together with the values for the chicks on standard oil, run in conjunction with them.

The results obtained by the two methods of assay on the eight vitamin D carriers are presented in Table XXII. Assay values presented were obtained by graphical methods as outlined.

Table XXI

Phosphatase Activity

I. Units Vit. D /100 gm. Ration	Standard Oil	Oil 59		
0	0.585			
4.4		0.484		
10.0	0.527	0.481		
15.0		0.405		
22.5	0.311	0.302		
33.75	0.349			
		Oil 60	Oil 61	Oil 62
0	0.766			
4.4	0.626	0.491	0.520	0.538
10.0	0.430	0.471	0.482	0.582
15.0	0.464	0.250	0.373	0.381
22.5	0.357	0.285	0.334	0.307
33.75	0.301	0.211		0.305
		Oil 63	Oil 70	
0	0.501			
4.4	0.407	0.446	0.468	
10.0	0.352	0.281	0.322	
15.0	0.358	0.244	0.196	
22.5	0.262	0.264	0.264	
33.75	0.257	0.178	0.189	
		Oil 71		
0	0.327			
4.4	0.413	0.448		
10.0	0.418	0.426		
15.0	0.351	0.285		
22.5	0.268	0.318		
33.75	0.264	0.334		
		Oil 72		
0	0.386			
4.4	0.394	0.362		
10.0	0.338	0.426		
15.0	0.297	0.284		
22.5	0.281	0.232		
33.75	0.253	0.261		

Table XXII

Results of Vitamin D Bioassays on Eight Oils using the Bone Ash Method and the Phosphatase Method.

Oil No.	Description	Bone Ash Assay	Phosphatase Activity
59	Pilchardine 400 D	300 D/gm	540 D/gm.
60	Sun-rendered 142 D	180 D/gm.	225 D/gm.
61	Shurgainoil 400 D	600 D/gm.	500 D/gm.
62	Frosst 30,000 D	35,000 D/gm.	34,400 D/gm.
63	Frosst 15-20,000 D	29,000 D/gm. 27,000 D/gm.	28,000 D/gm.
70	Flaydry 400 D	470 D/gm.	600 D/gm.
71	Flaydry 200 D	210 D/gm.	200 D/gm.
72	Flaydry 150 D	150 D/gm.	150 D/gm.

SUMMARY

Blood samples taken by bleeding directly from the severed neck into oxalated tubes saved time and labour and gave as good results as those obtained by the heart stab technique.

It has been found that a better representation of the phosphatase activity of a group of individuals is obtained by collecting individual samples and pooling equal amounts of blood from each, rather than obtaining a sample by pooling varying amounts of blood from individual chicks.

To avoid errors due to an increase or decrease in phosphatase activity in preserved samples, analyses should be carried out as soon as possible after sampling.

The pH optimum for the phosphatase of chick plasma using a 15-minute digestion and sodium β-glycerophosphate as the substrate was found to be pH 9.70 to 9.80.

The time of digestion was limited to 15 minutes in order to obtain the initial velocities of the phosphatase activity.

Since initial velocities are used and individual variation in phosphatase activity is very great, a low substrate concentration, namely 0.013 M. in the reaction mixture, was adopted for comparative studies of the effect of vitamin D deficiency on the activity of plasma phosphatase.

The optimum concentration of magnesium ion for maximum activity was found to be about 0.05 M, i.e. between 0.05M and 0.2 M (qMg 1.3 and 0.7).

The optimum concentration of glycine for maximum phosphatase activity was found to be 0.002 M. Starvation of the birds did not have

an appreciable effect on the optimum glycine concentration. In any case, the activation obtained was very slight and its role under the conditions of these experiments does not appear to be significant.

Dialysis of the plasma solution decreased the activity of the phosphatase very markedly, reducing it to a level at which measureable differentiation may be very difficult.

Manganese, while it activated the phosphatase in chick blood plasma, did not do so to the same extent as did magnesium, and further study would be required to establish its role in effecting optimum conditions of phosphatase activity.

The amount of oxalate present in the blood plasma solution failed to cause any significant inhibition, and this chemical was retained as the anti-coagulation agent in the sampling of the blood.

While slight hemolysis has no appreciable effect on the activity of the enzyme, complete hemolysis reduces the activity by about one-third.

In ordinary practical poultry raising, individual variation among chicks, with respect to blood plasma phosphatase activity, is high, even among progeny of the same dam. Intensive inbreeding as would be expected, leads to individuals with a much lower variation as regards blood plasma phosphatase activity and bone ash percentage.

The degree of relationship between the phosphatase activity of chick blood plasma and the dose of vitamin D and the relationship between the phosphatase activity and the tibia bone ash percentage reach their maximum when the chicks are four or five weeks old.

The Km of the phosphatase enzyme reaches a constant low value for

doses of vitamin D between 0 and 41.5 Units per 100 grams of feed when the chicks are six weeks old.

Magnesium ion concentration in the digest does not seem to have any effect on variations in the phosphatase Km.

Considering the 20% error allowed on biological assays, vitamin D bioassays on various D potent substances using this method seem to give fairly good results as compared with the regular A.O.A.C. chick method.

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II. SOME PRELIMINARY OBSERVATIONS ON THE CHEMICAL DETERMINATION OF VITAMIN D.

INTRODUCTION

In recent years there has been a growing interest in the possibility of devising a method for the chemical estimation of vitamin D in fish oils and other vitamin D carriers. Many chemists have made attempts to solve the problem and a great deal of information, as regards the chemical reactions of the vitamin, has been accumulated. However, little success has been achieved in the working out of a practical, chemical assay procedure. A preliminary survey of the literature has been made, and some initial investigations carried out on two of the more promising methods with a view to a possible furtherance of this work.

SURVEY OF THE LITERATURE

The procedures brought forth, to date, have been roughly classified on the basis of the reagents employed.

1. Antimony Trichloride Reagent

In 1936 Brockmann and Chen (1) published a method for the determination of vitamin D based on the colour reaction between vitamins D_2 or D_3 and a solution of antimony trichloride in chloroform. This colour is orange-yellow and shows a sharp absorption band at 500 millimicrons. It is claimed that the specificity of the reaction is very pronounced and that measurement of the band, the extinction of which is proportional the vitamin D concentration, may be used as a basis of determination. Tachysterol interferes but a large excess of other sterols and vitamin A has little or no effect. Extinction measurements are made ten to fifteen minutes after the addition of the reagent.

Emmerie and van Eekelen (3), using the method of Brockmann and Chen, obtained reliable results on the determination of calciferol and in a preparation of irradiated ergosterol in peanut oil. They found, however, that interfering substances must be taken into account.

According to Raoul and Meunier (15), who applied a modification of the Brockmann and Chen method colorimetrically, cholesterol, ergosteral, provitamins D_2 and D_3 as well as vitamins D_2 and D_3 give a colour reaction with antimony trichloride reagent. They found that cholesterol gave a slowly increasing reading and that vitamins D_2 and D_3 gave a rapidly decreasing reading. This reading was the intensity of absorption at 400 - 500 millimicrons.

Ritsert (16) maintains that the yellow colour produced by antimony trichloride with vitamins D_2 and D_3, varies in intensity with the age and sensitivity of the reagent. He concludes that the method is not suitable for use with oils containing vitamin A or a low concentration of vitamin D.

Numerous workers have tried to apply the Brockmann and Chen method but it is now known that, as a quantitative test, it is a failure (4).

Modifications in the antimony trichloride reaction have been published by Milas, Heggie and Raynolds (10) and by Nield, Russell and Zimmerli (13). Milas et al. after addition of the reagent, measure absorption at 620 millimicrons three minutes after mixing, at 500 - 520 millimicrons in ten to fifteen minutes, and at 480 millimicrons in thirty minutes. Using the first and last of these readings corrections may be applied to the $E^{1\%}_{1\ cm.}$ value at 500-520 millimicrons. If the colour intensity is to be measured in a photoelectric colorimeter, the interfering carotenoids, vitamin A and 7-dehydrocholesterol are destroyed by a preliminary treatment with maleic anhydride. The results of vitamin D assays on various high-potency fish oils, using this method, were in fair agreement with biological assay values. On low-potency preparations, the chemical assay results were very poor.

Using a modified antimony trichloride reagent, which included 2% acetyl chloride, Nield and co-workers determined, at 500 millimicrons, the optical density of colour produced with crystalline vitamins D_2 and D_3. The $E^{1\%}_{1\ cm.}$ value for the pure vitamin was found to be approximately 1800.

2. Pyrogallol and Aluminium Chloride.

When vitamin D is treated with pyrogallol and aluminium chloride, a lilac-red to reddish-violet colour develops. This reaction was utilized by Halden and Tzoni (6), (5), (21), as the basis of a colorimetric determination of the vitamin. Halden (5) states that cholesterol, ergosterol and luminosterol do not give the reaction. On the other hand, Tzoni (21) asserts that if sterols are present in large amounts they must be precipitated with digitonin before the colorimetric determination may be carried out. Tzoni's method also requires the quantitative removal of vitamin A, carotene, and "other reacting substances".

3. Bromine in Chloroform Solution.

The original Tortelli-Jaffe test (2) relied on the formation of a green colour when marine animal oils or their hydrogenation products were treated with a chloroform solution of bromine in the presence of acetic acid. In 1930, Heilbron and Spring (7) studied this reaction in relation to the structure of sterols. They believed it to be specific for sterol derivatives containing an "inert" (as applied to hydrogenation) linkage. They were unfortunate in choosing ergosterol as one of their test substances for in 1939 Westphal (22) showed that the Tortelli-Jaffe reaction was positive for a ring ditertiary double band. Two exceptions to this were the positive reactions of ergosterol and dihydroergosterol.

Through the use of this reaction, Solianikova (19) developed a quantitative photo-colorimetric determination of vitamin D. Four colour reactions of vitamin D were studied, the Tortelli-Jaffe reaction (20),

the Rosenheim reaction (17), the Liebermann-Burchard reaction (8) and the antimony trichloride reaction. Only the first test was found to be specific with absorption maxima at 545-550 and 590-600 millimicrons. Interferenoe, in the colorimetric determination, due to the presence of isomerides of vitamin D, may be eliminated by using light filters which absorb those parts of the spectrum where the absorption bands of these substances are situated. Consequently the estimation of the vitamin D-potency of natural or synthetic carriers may be made.

The latter claim, however, appears to be somewhat in error. Rutkovskii (18) has shown that the Tortelli-Jaffe reaction is not specific for irradiated ergosterol (vitamin D). Non-irradiated ergosterol reacts with Bromine in chloroform solution to give the same coloration and extinction value.

4. Other reagents.

Colour reactions of cholesterol, ergosterol, vitamin D and follicular hormone with a wide variety of reagents have been described in detail by Novak (14). Woker and Antener (23) have described a colour reaction of vitamin D_2 and ergosterol with alcoholic furfural solution in the presence of concentrated sulphuric acid.

5. Spectrography.

Spectrographic determinations of vitamin D have also been made without the use of any colour reaction. Nakamiya and Takizawa (11), measuring the extinction coefficient at 265 millimicrons of materials containing vitamin D or of the unsaponifiable fraction of these materials,

could find no relationship between this value and the vitamin D content as determined by bioassay. They found that saponification, while it did not affect the spectrographic determination, resulted in an actual loss of 20-30% of the vitamin D. Vitamin A interferes in the determination. Later Nakamiya and Koizumi (12) found that vitamin A was destroyed faster than vitamin D by exposure to ultra-violet light and that both were destroyed more rapidly in the unsaponifiable fraction than in the untreated oil. Similarly, dilution of the solution during irradiation increased the rate of destruction. $E^{1\%}_{1\ cm}$ values for pure vitamin D at 265 millimicrons averaged 462.

By a process of adsorption on hydraffin K 4 and elution with heptane, vitamin D may be separated from the unsaponifiable fraction of fish liver oils. The vitamin D in the eluate may be determined spectrographically. Marcussen (9), the originator of this technique, claims an accuracy of ± 7%. The $E^{1\%}_{1\ cm}$ value at 265 millimicrons obtained for pure calciferol, was 423.

The accuracy of the determination of the absorption spectrum of solutions containing vitamin D has been questioned by Crews and Smith (2). They claim that the extreme photo-lability of calciferol on exposure to ultra-violet light and even to visible light under certain conditions, necessitates special precautions. These workers have devised a continuous-flow method for measuring the ultra-violet absorption spectrum.

PRELIMINARY INVESTIGATIONS

Some preliminary investigation has been carried out on the method of Nield et al (13) with a view to the application of this technique to the photoelectric colorimeter.

A calibration curve for the intensity of yellow colour developed when Nield's antimony trichloride reagent was added to a chloroform solution of calciferol, was first attempted. It was found that when the proportion of standard solution to reagent was varied, the readings fluctuated widely. Examples of these results are found in Table I.

Nield's method calls for the determination of the optical density of the reaction mixture "within four minutes". Using a synthetic vitamin D_3 preparation in sesame oil solution, observations regarding the effect of time of reaction were recorded as shown in Table II.

Since little brownish colour developed during the first three minutes it was decided to adopt a three minute interval for the development of the yellow color with the standard solution as well as with unknown, test solutions.

Liver oils containing vitamin A could not be used in the determination until vitamin A was destroyed or removed. Attempts were made to destroy the vitamin by oxidation with various reagents. In all cases so far encountered, where oxidation was sufficiently severe to destroy vitamin A, the speed with which the antimony trichloride colour with vitamin D turned brownish, was markedly increased. The intensity of this brown discoloration made attempts to measure the absorption, at 500 millimicrons, impossible. The oxidizing agents employed were benzyl peroxide, maleic anhydride, dilute hydrogen peroxide and dilute nitric acid.

TABLE I

Effect of proportion of test solution to reagent on the intensity of color developed.

cc. of test solution	cc. of reagent.	vitamin D/cc reaction mixture	Colorimeter reading.
0.05	9.95	0.982	42.0
0.20	9.80	0.982	60.5
0.04	9.96	0.786	49.3
0.20	9.80	0.786	68.0

TABLE II

Effect of time of reaction on development of color intensity.

Test Solution	Time (min)	Colorimeter reading	
Unsaponified oil in $CHCl_3$	1	86.3	
	2	82.0	
	3	80.5	
	4	79.0	
	5	78.0	
	6		color rapidly turning brownish
	7	77.5	
	8		
	9	75.5	
Unsaponifiable fraction in $CHCl_3$	1	86.0	
	2		
	3	84.5	
	4		
	5	83.5	color slowly turning brownish

Oxidation of the carotenoids was also carried out utilizing the bubbling of air through a solution of the oil or of the unsaponifiable fraction. A similar increase in the rate of browning of the reaction mixture occurred.

A few experiments were made on the Tortelli-Jaffe reaction as utilized by Solianikova (19) with no encouraging results. In his paper, Solianikova fails to describe his technique in much detail. No mention is made of the strengths of the solution employed and the duplication of his results would require a very intensive investigation. It has been found that the green colour, developed when fish oils are treated with bromine-chloroform solution, fades slowly after a preliminary intensification. This green colour failed to show any marked absorption peak at 550 or 600 millimicrons, the bands described by Solianikova. The proportion of bromine solution to oil necessary for maximum development of the green colour appears to vary from one oil to another.

CONCLUSIONS

Until some feasible method for the easy removal of the interfering substances in fish liver oils is discovered, it is felt that the reaction with antimony trichloride cannot be employed with any marked degree of success.

On the basis of the preliminary investigations carried out, it would appear that the use of the Tortelli-Jaffe reaction, as a basis for the quantitative determination of vitamin D in naturally occurring substances, showed the greatest possibilities of the methods so far proposed.

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