Studies on the Plasma Phosphatase of Normal and Rachitic Chicks

1. GENERAL CHARACTERISTICS OF THE ENZYME

By I. MOTZOK, *Department of Animal Nutrition, Ontario Agricultural College, Guelph, Canada* and A. M. WYNNE, *Department of Biochemistry, University of Toronto, Toronto, Canada*

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The primary purpose of these investigations was to determine whether or not there is a quantitative relationship between the level of plasma phosphatase in rachitic chicks and the severity of the rachitic conditions as ordinarily expressed in terms of bone ash.

It is now generally recognized that rickets and other disturbances of the normal process of bone formation are usually accompanied by an increase in the alkaline-phosphatase activity of the blood plasma. The literature bearing on this relationship has been reviewed by Folley & Kay (1936) and, more recently, by Morris (1944) and by Moog (1946). Others, including Bodansky & Jaffe (1934*a*, *b*), Barnes & Carpenter (1937) and Klasmer (1944) have reported clinical studies which support the view that the degree of activity of the alkaline phosphatase of the plasma is directly related to the severity of the rachitic condition. Elevated plasma phosphatase is one of the earliest changes to be observed during the development of rickets in children, and the diminution in the concentration of the enzyme in the plasma towards normal levels, during treatment of the disease, is regarded as good evidence of the healing of rickets.

In the case of the chick, it has been established that the mineral content of the bones bears a direct relationship, within certain limits, to the amount of vitamin D consumed with the food; and, as is well known, determination of the ash content of the tibiae serves as the basis of a method (Association of Official Agricultural Chemists, 1940) of assaying the potency of antirachitic substances used in poultry feeding. Although this method is widely used, the preparation of the bones for the determination of their ash content is a rather tedious and time-consuming process.

Before undertaking the studies of the quantitative relationship between the level of plasma phosphatase in rachitic chicks and the severity of the disease as indicated by the bone ash, it was necessary to investigate the properties of the alkaline phosphatase of chick plasma in order to establish the optimum conditions for its activity *in vitro* and to develop a reliable method for the quantitative determination of the enzyme. Surprisingly little information on the characteristics of this enzyme in chicks is available in the literature. It is true that several investigators have established a correlation between the degree of rickets in chickens and the amount of plasma phosphatase expressed in terms of its activity (Auchinachie & Emslie, 1934; Common, 1936; Correll & Wise, 1938), but the methods used for the measurement of the activity of the enzyme were those which had been employed previously by other workers in studies of the phosphatases of mammalian sera and tissue extracts. There is, however, no reason for assuming that the optimum conditions for the activity, *in vitro*, of the plasma phosphatase of chicks are identical with those found for the phosphatases of mammalian species. Indeed, Bakwin & Bodansky (1933) have shown that the same type of tissue, e.g. intestinal mucosa, obtained from different mammalian species may yield phosphatase preparations which require somewhat different conditions for their optimum activity *in vitro*.

In view of these considerations, some preliminary studies of the chick enzyme were carried out, with the purpose of establishing optimum conditions for its activity. The present paper is concerned with these studies.

EXPERIMENTAL AND METHODS

Single comb White Leghorn chicks were started on experiment when 1 day old. The chicks were divided into groups of twenty or more and kept in metal chick batteries in an air-conditioned room maintained at approximately 27°. They were fed *ad lib.* for 3 weeks, on the basal diet recommended by the Association of Official Agricultural Chemists (1940) for the chick assay of vitamin D, fortified by graded amounts of cod liver oil previously assayed for vitamin D by the A.O.A.C. procedure.

Blood samples. After 3 weeks, individual samples of blood from the carotid artery were collected in dry glass vials containing Na oxalate. Equal portions (e.g. 0·5 ml.) of blood drawn from individual chicks were pooled to make a representative sample for the group. In the early experiments 10 ml. of the pooled blood were added to 200 ml. of 0·9% (w/v) NaCl solution; the mixture was subsequently centrifuged and the clear plasma solution was removed. A few drops of toluene were added as a preservative and the saline plasma solution was kept at 2·5°. The phosphatase activity of such a preparation was found to be unstable, as will be shown later; therefore, in studies of the characteristics of the enzyme present in different samples of blood, prepared by the method just described, the activity was determined 1 hr. after dilution. It was established later that, if the plasma was stored at 2·5° and diluted with saline just before use, the enzyme was stable for at least 2 weeks. This method of preparation of the enzyme was used when comparative studies, extending over several days, were made on the same pooled sample of blood.

Substrate. In most of the experiments Merck's N.F. disodium glycerophosphate ($Na_2C_3H_5(OH)_2.PO_4.5\frac{1}{2}H_2O$; mol.wt. 315·15) was used as the substrate. It was found to be free from inorganic phosphate. During the latter part of the investigation the analysis of this preparation by a modification* of the methods of Malaparade (1928) and of Voris, Ellis & Maynard (1940) showed that it contained approximately 33% of α-glycerophosphate and 67% of the β-salt. Throughout the paper this mixed salt will be referred to as sodium glycerophosphate. In certain other experiments a preparation of the pure β-salt was used; this is designated as β-glycerophosphate.

Buffer. In almost all the studies reported in this paper the veronal-carbonate buffer of King & Delory (1940) was used. After some preliminary studies (Graham, 1942; Motzok, unpublished data) with the NH_4OH-NH_4Cl buffer recommended by Lundsteen & Vermehren (1936), it was established, as will be shown later, that the activity of the enzymes at a given pH varied with the concentration of the buffer in the digestion mixture. With the veronal-carbonate buffer, on the other hand, under the conditions employed and at a given pH, variation in the concentration of the buffer in the digestion mixture from 0·0025 to 0·025 M did not cause any change in the activity of the enzyme. In all but one of the experiments the concentration of this buffer in the reaction mixture was 0·0125 M.

Determination of phosphatase activity. 10 ml. of the saline plasma solution (1 ml. of plasma diluted with 19 ml. of saline) were added to 10 ml. of a solution containing substrate, buffer and $MgCl_2$, the two solutions having previously been warmed to 30°. After thorough shaking, the reaction mixture was incubated for 15 min. at 30°. The action of the enzyme was stopped by trichloroacetic acid (4 ml. of digestion mixture were added to 3 ml. of 15% (w/v) trichloroacetic acid in a centrifuge tube), and the liberated inorganic phosphate in the supernatant solution after centrifuging was determined by the method of Lundsteen & Vermehren (1936), with the modification that, instead of amidol, the reducing agent used was 1-amino-2-naphthol-4-sulphonic acid prepared by the method of King (1932). The density of the colour developed was determined with a Cenco-Sheard-Sanford photelometer. Control mixtures containing enzyme, buffer and $MgCl_2$ but no substrate were treated similarly. In other mixtures containing substrate, buffer and $MgCl_2$ but no enzyme, no hydrolysis of the substrate took place under the conditions adopted.

Bodansky (1932) has shown that an appreciable error may be incurred in the estimation of inorganic phosphate in the presence of varying concentrations of glycerophosphate and of trichloroacetic acid. In the present studies sufficiently small samples were used to avoid any interference by glycerophosphate or by acid and also to eliminate the necessity of neutralization with NaOH as proposed by Shinowara, Jones & Reinhart (1942). The limits of error between duplicate phosphatase determinations were within ±2%. By means of a glass electrode the pH of each reaction mixture was determined at the end of the digestion period, at room temperature (approx. 25°), and is designated as the 'final' pH. It was found that no appreciable change in the pH occurred during the period of hydrolysis.

Unless otherwise stated, plasma phosphatase activity is expressed in terms of inorganic phosphate, recorded as mg. of phosphorus liberated from Na glycerophosphate by the enzyme present either in 0·5 ml. of cell-free plasma or in the plasma of 1 ml. of blood.

RESULTS

Effect of buffers. The inhibitory effect of ammonium buffer was shown by a fall from 1·37 mg. P hydrolysed to 1·06 mg. as the concentration of buffer was raised from 0·005 to 0·04 M, the pH being constant at 9·88 with 0·02 M-Na glycerophosphate and 5 mM-$MgCl_2$. Although a constant concentration of NH_4^+ can, of course, be maintained in the digestion mixture, the quantity of enzyme present may vary from one sample of blood to another, resulting in a corresponding variation in the ratio of the enzyme to NH_4^+. Under such conditions the inhibitory effect of the NH_4^+ would be a variable factor.

Variations in the concentration of veronal-carbonate buffer in the reaction mixture had no effect on the activity of the enzyme. Between 0·0025 and 0·05 M the P hydrolysed varied between only 1·17 and 1·19 mg.

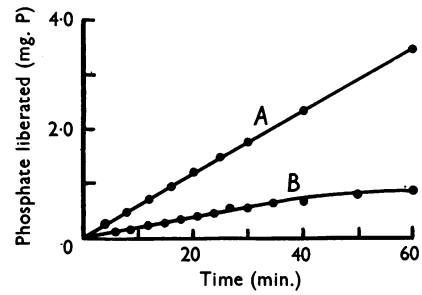

Fig. 1. Time course of hydrolysis of 0·08 M-Na glycerophosphate at pH 9·88 and in the presence of 0·005 M-$MgCl_2$ (*A*), and of 0·06 M-Na glycerophosphate at pH 9·86 without added Mg^{++} (*B*), by plasma phosphatase of chicks.

Initial velocity of reaction. In an effort to establish a convenient reaction time within which the initial velocity of the reaction remains constant, a number of experiments of the type of that illustrated in Fig. 1 were carried out. From this figure it is clear that, under the conditions specified, the initial velocity is constant for at least 30 min. even without added magnesium chloride (curve *B*); and, with added magnesium chloride, linearity is maintained for at

* The authors are indebted to Dr C. S. McArthur, Banting and Best Department of Medical Research, University of Toronto, for details of his modification of the method of analysis. The modifications specified that the phosphate buffer should consist of 17 g. of $Na_2HPO_4.12H_2O$ and 4·2 ml. of 10 N-H_2SO_4 in 100 ml. of solution, that a reaction period of 3 hr. be used and that a saturated aqueous solution of phenolphthalein be employed as the indicator instead of $MgSO_4$.

least 60 min. (curve *A*). On the basis of these experiments, it was concluded that determinations of inorganic phosphate made after 15 min. hydrolysis can safely be regarded as representing initial velocities of reactions.

Relationship between substrate concentration and optimum pH. The concentration of substrate in the reaction mixture was shown to have a rather marked influence on the pH required for optimum activity of the enzyme. Fig. 2 indicates that the optimum pH shifted from a value of 9·65 for the hydrolysis of 0·004 M-sodium β-glycerophosphate to pH 9·88 for

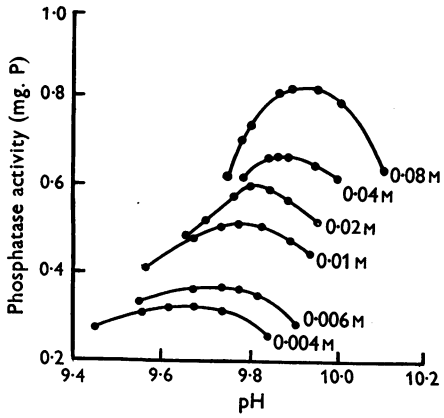

Fig. 2. Relationship between pH and the hydrolysis of different concentrations of Na β-glycerophosphate by chick plasma phosphatase in the presence of 0·005 M-$MgCl_2$ during a reaction period of 10 min.

the hydrolysis of 0·08 M-substrate, in mixtures containing 0·005 M-magnesium chloride. Similar changes in the optimum pH in relation to substrate concentration were observed in the case of the mixture of α- and β-glycerophosphate in the presence of added magnesium chloride and in the case of the pure β-ester without additions of magnesium chloride. This variation in optimum pH with change in initial substrate concentration is in general agreement with the more limited findings of Askawa (1928), Jacobsen (1933) and Folley & Kay (1935), and has been confirmed by Shen (1943) and Emmett (1944) for the phosphatases of yeast and of the intestinal mucosa of rats, respectively.

There is much confusion in the literature with respect to the pH optima of alkaline phosphatases. The range of reported optima extends from values at least as low as pH 8·2 to values as high as pH 10 or higher. While there is no doubt that phosphatase derived from different tissues of the same animal may be characterized by different pH optima and that different species of animals may exhibit differences with respect to the optimum pH of the phosphatase derived from similar organs or tissues, it is nevertheless true that confidence in reported values is necessarily determined by considerations of the methods by which these values were obtained. As Folley & Kay (1936) have suggested, much of the confusion may be traced to the failure of some workers to use the initial velocity of reaction as the basis of comparison of the activities of the enzyme under different conditions. (The only satisfactory alternative is the use of the unimolecular velocity constant, but only under certain conditions can K_{uni} be shown to be really constant over an appreciable period of time.) The arbitrary choice of a reaction period at the end of which the products of the reaction are measured is open to serious criticism, for, depending upon this choice, different apparent optimum pH values may be obtained. The work of Lundsteen & Vermehren (1936) on the alkaline phosphatase of human serum illustrates this point. For long periods of hydrolysis (70 hr.) they reported an optimum pH of 8·65, whereas for periods of 1 hr. greatest activity was observed at pH 9·65. No information was given as to change of pH during the 70 hr. period, but it is unlikely that the pH was constant. In any event, the concentration of substrate was considerably reduced during the later stages of this long period; and since the optimum pH varies to some extent with the substrate concentration, it is difficult, if not impossible, to reach any valid conclusion as to the true optimum pH of such a system. It should be emphasized that reliable values for the optimum pH of phosphatases, as indeed of enzymes in general, are usually obtainable only when activities are compared on the basis of initial rates of reaction. Moreover, in the case of phosphatases it is desirable, when recording optimum pH values, to state also the initial concentration of substrate since variations in this concentration undoubtedly affect the optimum pH.

Optimum concentration of substrate. Previous studies of the relationship between substrate concentration and phosphatase activity have been reviewed by Folley & Kay (1936). Numerous workers have observed an increase in the initial rate of hydrolysis which rises to a maximum value with increasing substrate concentration; and with concentrations of substrate above the 'optimum', a progressive falling off in the rate of hydrolysis has frequently been observed. This diminution in activity has been attributed to 'inhibition by excess substrate'. It should be noted that previous workers, in studying the relation between substrate concentration and phosphatase activity, have adjusted all reaction mixtures (containing increasing concentrations of substrate) to the same initial pH, namely an 'optimum' pH value obtained by measuring the activity of the enzyme in reaction mixtures adjusted to different pH levels and containing an arbitrarily chosen concentration of substrate.

The alkaline phosphatase of chick plasma has been found in the present study to behave in a similar manner under the conditions just outlined. For example, when the pH of reaction mixtures containing sodium glycerophosphate in different concentrations was maintained at the same level in all cases, namely, at pH 9·86 (the optimum pH for 0·06 M-substrate) the initial rate of hydrolysis increased with increasing concentration of substrate

up to a maximum rate obtained with 0·06 M-substrate; with higher concentrations of substrate a progressive decrease in the initial rate of hydrolysis occurred. Since the optimum pH has been shown to vary with the initial concentration of substrate there would seem to be no good reason for selecting, arbitrarily, a single pH level at which the activity of the enzyme in mixtures containing different concentrations of substrate is to be measured. If, instead, the several activities are measured at the pH levels which have been found to be optimum for the different concentrations of glycerophosphate employed, no diminution in activity is observed in the higher ranges of concentration at least up to 0·10 M (Fig. 3). The accurate determination of the plasma-

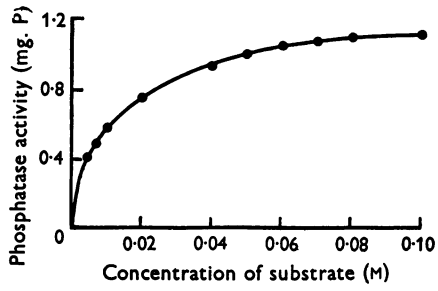

Fig. 3. Effect of substrate concentration on the rate of hydrolysis of Na glycerophosphate by chick-plasma phosphatase at the optimum pH for each concentration of substrate in the presence of 0·005 M-$MgCl_2$ during a reaction period of 10 min.

phosphatase activity in mixtures containing glycerophosphate in concentrations greater than 0·10 M is complicated by the difficulty of maintaining the pH at the optimum levels, since these lie close to the effective limit of the buffer system employed.

Magnesium activation of plasma phosphatase. The maximum activation of chick-plasma phosphatase by Mg^{++} in the hydrolysis of 0·08 M-sodium glycerophosphate, during a reaction period of 15 min., was obtained with 0·005 M-magnesium chloride when the pH was maintained at 9·88–9·90. Further additions of magnesium chloride up to 0·02 M had no effect on the rate of hydrolysis; concentrations above 0·02 M resulted in precipitation of magnesium hydroxide and a lowering of the pH below the optimum pH 9·90. The optimum concentration of Mg^{++} was not affected appreciably by a change in concentration of enzyme, e.g. from 0·5 to 0·25 ml. of plasma per 20 ml. of digestion mixture.

Stability of plasma phosphatase. The results of a study of the stability of the enzyme at 2·5° are illustrated in Fig. 4 in which the two curves, *A* and *B*, represent two preparations made by slightly different methods from pooled samples of freshly drawn oxalated blood. One preparation was obtained by diluting 1 vol. of blood with 20 vol. of physiological saline, and subsequently removing the cells by centrifugation. A small amount of toluene was added. Curve *A* of Fig. 4 shows that the alkaline phosphatase activity of this preparation decreased rapidly during the first 10 hr. of storage at 2·5°, after which the rate of inactivation was slower. In contrast, the activity of the other preparation, undiluted cell-free plasma, did not change appreciably during at least 2 weeks' storage at 2·5° (curve *B*).

If the cells are removed from the undiluted blood and the plasma is subsequently diluted with 20 vol. of 0·9 % (w/v) sodium chloride solution, the phosphatase activity of the diluted plasma remains unchanged for at least 1 hr. at 30°; in sixteen successive

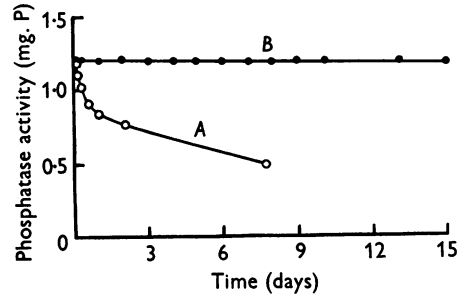

Fig. 4. Stability of phosphatase activity of plasma of rachitic chicks when the enzyme preparations were stored at 2·5°. *A* was obtained with oxalated blood diluted with saline prior to removal of cells by centrifugation, and *B* was obtained with cell-free plasma diluted with saline 15 min. prior to determination of activity; 0·08 M-Na glycerophosphate, pH 9·88, 0·005 M-$MgCl_2$ and time of hydrolysis 15 min.

samples, taken at intervals of 3 or 4 min., the phosphorus liberated varied only between 0·502 and 0·509 mg., during a reaction period of 5 min. at 30°.

In the light of the results of these experiments on the stability of the enzyme, the following standard procedure for the preparation of the enzyme was adopted whenever comparative studies, extending over a period of a day or two, were to be made on the same pooled sample of oxalated blood; the cells were removed by centrifugation, the undiluted plasma was stored at 2·5° until required, and the activity was determined not later than 30 min. after diluting the plasma with saline.

Effect of sodium chloride and of sodium oxalate on phosphatase activity. When the phosphatase activity of the plasma is at a high level, as in the case of severely rachitic chicks, it is convenient to work with smaller volumes of the saline plasma solution than those employed in studies of less active plasma. If the same volume of final reaction mixture is employed in all cases it is necessary to determine

whether or not sodium chloride, present in the reaction mixture in different concentrations, influences the phosphatase activity of the system. It was found that the activity is not affected by variation in the concentration of the salt between 0·13 and 1·72% (w/v) in the reaction mixture, the phosphorus liberated at ten different concentrations varying only between 0·770 and 0·790 mg.

In studies of the influence of oxalate, Belfanti, Contardi & Ercoli (1935*a*, *b*) found no effect on the alkaline phosphatase of rabbit liver and kidney, but they observed considerable inhibition of the alkaline phosphatase of horse and rabbit sera. Bodansky (1933) calculated the average phosphatase activity of oxalated plasma to be 90% of the activity of serum. Auchinachie & Emslie (1934), on the other hand, could find no significant difference in phosphatase activity between the serum and oxalated blood plasma of chickens.

We have found that sodium oxalate, in concentrations up to 0·00625 M in the reaction mixture, had no appreciable effect on the activity of the plasma phosphatase of rachitic chicks, the phosphorus liberated varying only between 0·98 and 1·01 mg. at nine different levels in a particular experiment. The plasma was prepared by collecting arterial blood from twenty chicks directly into saline solution containing no oxalate, and removing the cells by centrifugation. The oxalate was added to the digestion mixture, and the activity of the enzyme was measured under conditions which were optimum with respect to the concentration of substrate and of Mg^{++} and H^{+}. In all the other studies reported in this paper the concentration of oxalate in the reaction mixture fell within the range 0·0005–0·001 M.

Effect of dilution on the activity of the enzyme. The studies reported thus far were designed to establish the optimum conditions for the activity of the enzyme. But before adopting any set of conditions for the routine assay of plasma for phosphatase activity, it is essential that under these conditions the activity of the enzyme in the reaction mixture shall bear a direct linear relationship to the concentration of the enzyme.

This relationship was studied with an enzyme preparation obtained from a group of chicks which had received 10 units of vitamin D per 100 g. of ration, the plasma having a relatively high concentration of enzyme. The cells were removed by centrifuging the pooled oxalated blood, different volumes of plasma were measured accurately and diluted to 40 ml. with saline, and the hydrolysis was carried out, as described previously, in reaction mixtures containing 0·08 M-Na glycerophosphate and 0·005 M-$MgCl_2$ at pH 9·88 and 30° during a reaction period of 15 min.

A linear relationship was found between the activity of the system and the concentration of the enzyme up to a concentration of 0·75 ml. of undiluted plasma in 20 ml. of digestion mixture. In most of the studies reported in this paper, the volume of saline plasma present in each reaction mixture of 20 ml. represented 0·25 ml. of undiluted plasma. In no case did the volume of diluted plasma in the reaction mixture represent more than 0·75 ml. of original plasma.

Effect of vitamin D in the diet of the chick on the optimum conditions for plasma phosphatase activity. In the experiments hitherto discussed, representative samples of plasma were obtained from chicks reared on diets consisting either of the basal ration alone or of the basal ration supplemented with vitamin D in amounts up to 20 units per 100 g. of ration. In view of the fact that vitamin D deficiency has been shown to be associated with abnormalities in the composition of the blood, it is necessary to know, if one wished to compare the plasma phosphatase of rachitic chicks with that of chicks fed vitamin D in the diet, whether these abnormalities have any influence on the optimum conditions for the activity of the enzyme *in vitro*.

For the purpose of obtaining this information two lots of plasma were prepared from the blood of two groups of forty chicks each. One group had received no vitamin D supplement and the other 20 units of vitamin D_3 per 100 g. of basal ration during a period of 3 weeks extending from 1 day of age to the time of the preparation of the plasma. The phosphatase activity of the two preparations was investigated in relation to the following factors: (1) influence of pH on the activity in mixtures containing 0·08 M-Na glycerophosphate and 0·005 M-$MgCl_2$; (2) influence of substrate concentration in mixtures containing 0·005 M-$MgCl_2$ and adjusted to the pH optima previously established for the several substrate concentrations employed; (3) influence of $MgCl_2$ in various concentrations, in mixtures containing 0·08 M-Na glycerophosphate and adjusted to pH 9·88.

No significant difference between the two preparations with respect to any of the factors mentioned was observed, although the activity of the rachitic plasma was found to be considerably higher than that of the plasma of chicks which had received vitamin D, when the two activities were measured under the same conditions. It was concluded that the optimum conditions previously established can safely be adopted in comparative studies of the activity of the enzyme of rachitic chicks and of those which receive supplements of vitamin D in their diet.

The possibility of diurnal variation in phosphatase activity. Since blood samples were usually collected between the hours of 9 a.m. and 3 p.m. it was desirable to know whether the phosphatase activity of the morning samples might differ from that of the afternoon preparations.

Two groups of chicks were used in this study; one group of chicks received no vitamin D supplement, while another group received a supplement of 10 units of vitamin D per 100 g. of the basal diet. At the end of 3 weeks one-half of the chicks in each group were killed at 9 a.m. and the other half at 3 p.m. The percentage ash of the pooled tibiae from each half of the two groups was determined in order to

serve as a check in considering any differences in the phosphatase activity of the plasma, but there was no significant difference in ash content.

There was also no difference between the phosphatase activities of the preparations obtained, the activity being 0·873 and 0·885 for the morning samples of the two groups, and 0·888 and 0·860 for the afternoon samples. This observation was not unexpected, in view of the fact that food was continuously available to the chicks from 6 a.m., the time at which the lights were turned on, until the time at which the chicks were killed.

SUMMARY

1. Plasma-phosphatase activity was inhibited by ammonium hydroxide-ammonium chloride buffer system to a varying degree depending on the concentration of buffer. On the other hand, variation in the concentration of the veronal-carbonate buffer from 0·0025 to 0·025M in the reaction mixture had no effect on the activity of the enzyme.

2. The rate of hydrolysis of 0·08M-sodium glycerophosphate at the optimum pH 9·88 and in the presence of 0·005M-Mg^{++} was found to be linear for at least 60 min., while the hydrolysis of 0·06M-substrate at the optimum pH 9·86 without added magnesium chloride was linear for only 30 min.

3. The optimum pH for the activity of plasma phosphatase was shown to be dependent on the concentration of substrate in the reaction mixture. With pure sodium β-glycerophosphate, in the presence of 0·005M-magnesium chloride, the optimum shifted from pH 9·65 for the hydrolysis of 0·004M-substrate to approximately pH 9·88 for the hydrolysis of 0·08M-substrate.

4. The activity of plasma phosphatase appears to be maximal when the substrate concentration is about 0·08M in the reaction mixture.

5. The concentration of Mg^{++} in the reaction mixture required for maximum activation of chick-plasma phosphatase for the hydrolysis of 0·08M-sodium glycerophosphate at the optimum pH was found to be 0·005M. Further additions up to 0·02M had no effect on the maximum rate of hydrolysis. The optimum concentration of Mg^{++} was not affected by the concentration of enzyme in the digestion mixture.

6. The activity of plasma phosphatase was found to decrease very rapidly when the blood was diluted with saline before the cells were removed by centrifugation, and the saline plasma solution stored at 2·5°. However, when the cells were removed from the plasma before dilution with saline, no loss in enzyme activity was evident for at least 2 weeks' storage at 2·5°.

7. Concentrations of sodium chloride, varying from 0·135 to 1·72% (w/v) in the reaction mixture, had no appreciable effect on plasma-phosphatase activity under the optimum conditions of pH, substrate and Mg^{++} concentration. Sodium oxalate, in concentrations up to 0·00625M in the reaction mixture, similarly had no effect on the activity of the enzyme under optimum conditions.

8. A linear relationship was found to exist between activity and concentration of enzyme at least as high as 0·75 ml. of plasma per 20 ml. of digestion mixture. In studies on the optimum conditions for plasma-phosphatase activity 0·25 ml. of plasma were used in 20 ml. of reaction mixture.

9. The previously established conditions for optimum activity of plasma phosphatase were not affected by the absence or presence of vitamin D in the chicks' ration. The phosphatase activity of the plasma of chicks which received no vitamin D in their diet was found to be considerably higher than that of chicks which received 20 units of vitamin D per 100 g. of ration.

10. No appreciable difference in activity of plasma phosphatase could be found during the period of the day in which the blood samples were collected, i.e. 9 a.m. to 3 p.m.

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Studies on the Plasma Phosphatase of Normal and Rachitic Chicks

2. RELATIONSHIP BETWEEN PLASMA PHOSPHATASE AND THE PHOSPHATASES OF BONE, KIDNEY, LIVER AND INTESTINAL MUCOSA

BY I. MOTZOK
Department of Animal Nutrition, Ontario Agricultural College, Guelph, and Department of Biochemistry, University of Toronto, Canada

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During the course of a study (Motzok & Wynne, 1950) of the alkaline phosphatase of chick plasma it was shown that the phosphatase activity of the plasma of chicks reared on a diet containing no vitamin D was greater than the activity of the plasma obtained from chicks which received supplements of vitamin D in addition to the basal diet.

There is much evidence which suggests that in these rachitic disorders the increase in the activity of plasma phosphatase is, in general, directly related to the severity of the condition (see Moog, 1946). The interpretation of the increased activity of the enzyme in the plasma phosphatase as evidence of increased concentration of the enzyme in the plasma, however, has been questioned by Thannhauser, Reichel, Grattan & Maddock (1938), who suggest that the abnormally high levels of activity may be due to the effects of an activator which is present in abnormal, but not in normal, plasma. That this is the true explanation seems unlikely, in view of the work of Williams & Watson (1940, 1941), of Delory & King (1944), and of Gould (1944). The majority of workers support the view that relative phosphatase activities can safely be interpreted in terms of relative concentrations of enzyme, provided that the activities are measured under standardized and optimum conditions. Unfortunately this last provision has not always been fulfilled.

The problem of the origin of the increased phosphatase content of the plasma in pathological conditions of various kinds has been extensively investigated. No attempt will be made in this paper to review the literature relating to this problem; valuable discussions of its several aspects have been contributed by Robison (1932), Kay (1933), Folley & Kay (1936), Jaffe & Bodansky (1943), Morris (1944) and Moog (1946).

Many investigations have shown that alkaline phosphatase is present in high concentrations in the epiphysial zone and periosteum of bones and in the intestinal mucosa and kidneys, while the liver usually contains a relatively much smaller amount of the enzyme. The present communication presents evidence that the increased alkaline plasma phosphatase of rachitic chicks is of skeletal origin, that the amounts of alkaline phosphatase in the intestinal mucosa, kidney and liver are not related to the vitamin D content of the diet, and that it is unlikely that these three tissues constitute the source of any appreciable amount of the elevated plasma phosphatase in rachitic chicks.

EXPERIMENTAL

As stated previously (Motzok & Wynne, 1950), the primary purpose of these investigations was to determine whether or not, in rachitic chicks, a quantitative relationship exists between the level of plasma phosphatase on the one hand, and the severity of the rachitic condition as ordinarily expressed in terms of bone ash, on the other. In order to obtain trustworthy information with respect to the amount of phosphatase in the plasma, for subsequent use in the development of a method for the assay of vitamin D preparations, it was necessary to establish as carefully as possible the optimum conditions for the activity and stability of the enzyme *in vitro*. On the other hand, for the purpose of determining whether or not changes in the level of dietary vitamin D have any appreciable influence on the phosphatase content of the tissues it was considered sufficient to establish only the more important conditions for the optimum activity of the enzymes from these sources. Accordingly, the optimum pH and Mg^{++} concentration were determined for the hydrolysis of a relatively high concentration of Na glycerophosphate (0·05 M), and then, using these optimum values, the concentration of substrate required for maximum activity was determined.

Single-comb White Leghorn day-old chicks were used in all experiments. They were fed, *ad lib.*, for 3 weeks on the basal diet recommended by the Association of Official Agricultural Chemists (1940) for the chick assay of vitamin D, supplemented by graded amounts of cod liver oil previously assayed for vitamin D. The amounts of vitamin D added to the basal diet were 0, 10, 20 and 40 units/100 g. of ration. The recommended allowance of vitamin D for chicks