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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

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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 epiphyseal 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 (0.05M), 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

is 40 units/100 g. of ration (U.S.A. National Research Council, 1944). At the end of the feeding period at least twenty chicks in each dietary group were killed by decapitation and blood samples were collected. The liver, kidneys, small intestines and one tibia were removed from each chick; the tissues were pooled and extracted in the manner described below and phosphatase activities were determined immediately after preparation of the extracts.

Preparation of tissue phosphatase

Bone. The tibiae were freed from adhering tissue, weighed in a weighing bottle, and subsequently cut into small pieces which were further broken up in a Waring blender containing about 200 ml. of distilled water. The suspension was then diluted to such a volume that 100 ml. contained 1 g. of bone. After allowing the coarse material to settle out, the suspension was centrifuged; the supernatant liquid was siphoned off and used for phosphatase determinations.

Intestine. Only the first half of the duodenum was used; the contents were washed out with a stream of distilled water, the sections were turned inside out and washed with four changes of 0.9% saline, followed by one washing with distilled water. The mucosa was removed with a bone spatula, thoroughly dispersed in distilled water in a Waring blender, and finally diluted to give a concentration of 1 g. of mucosa (dry weight basis) per litre of suspension.

Kidney and liver. The method of preparation of the enzyme extracts was the same for both organs. They were weighed in weighing bottles immediately after removal, washed once with distilled water, and macerated in distilled water in a Waring blender. The suspensions were then diluted to such volumes that 100 ml. contained 1 and 2 g. (wet wt.) of kidney and of liver, respectively. The suspensions were centrifuged and the supernatant solutions, containing the enzyme, were siphoned off.

Determination of phosphatase activity

The method for the determination of plasma phosphatase activity, which has been outlined previously by Motzok & Wynne (1950), was used for the determination of the phosphatase activity of the various tissues. Disodium glycerophosphate ($\text{Na}_2\text{C}_2\text{H}_3(\text{OH})_2\text{PO}_4 \cdot 5\frac{1}{2}\text{H}_2\text{O}$, mol. wt. 315.15) which was found later to contain approximately 33% of the α -ester and 67% of the β -ester, was used in all the investigations reported in this paper. The activities of tissue phosphatase are expressed as mg. of inorganic P liberated in 15 min. at 30° from 0.05 M-Na glycerophosphate in mixtures containing the optimal concentrations of Mg^{++} at the optimum pH (measured at room temperature), by the enzyme in extracts corresponding to 1 g. (wet wt.) of bone, kidney or liver and to 1 g. (dry wt.) of intestinal mucosa. In all cases the initial zero order rates of reaction were determined.

RESULTS AND DISCUSSION

Properties of the phosphatases of bone, kidney, liver and intestinal mucosa

Optimum pH. The influence of pH on the activity of the phosphatase of intestinal mucosa, bone, kidney and liver tissue is illustrated in Figs. 1 and 2, curves A-D, respectively. The optimum pH for bone, kidney and liver phosphatases (pH 9.85) is

identical with that previously found for the plasma phosphatase (Motzok & Wynne, 1950), when the concentration of glycerophosphate in the digestion mixture is 0.05 M in all four cases. On the other hand, the phosphatase of intestinal mucosa exhibits greatest activity at a more acid pH, namely, pH 9.20 for 0.05 M-substrate. The optimum range of pH for kidney and liver phosphatase was wider than that for the enzyme of bone and much wider than that for the enzyme of intestinal mucosa.

Fig. 1. Effect of pH on the hydrolysis of 0.05 M-Na glycerophosphate by the phosphatase of intestinal mucosa (A), and bone tissue (B) of rachitic chicks in the presence of 0.01 M- and 0.005 M- MgCl_2 , respectively, during a reaction period of 15 min.

Fig. 2. Effect of pH on the hydrolysis of 0.05 M-Na glycerophosphate by the phosphatase of kidney tissue (C), and liver tissue (D) of rachitic chicks in the presence of 0.005 M- MgCl_2 during a reaction period of 15 min.

On the basis of the pH optima, one may conclude that it is unlikely that the rise in the level of plasma phosphatase associated with rickets is the result of 'leakage' of the enzyme from the intestine, but that the bone, kidney or liver, or all three tissues, might be the source of the increase of the enzyme in the plasma.

Optimum concentration of Mg ions. Maximum activity of the phosphatase of intestinal mucosa was observed in mixtures containing 0.01M-Mg⁺⁺. Increasing the concentration of Mg⁺⁺ up to 0.02M did not change the rate of hydrolysis of 0.05M-glycerophosphate at pH 9.20. These findings are in general agreement with those of Bakwin & Bodansky (1933) and of Macpherson (1942), who used rats. It should be kept in mind, however, that the concentration of substrate used in the present study may not be optimum for the phosphatase of the intestinal mucosa of rats. The agreement of these data with those obtained by workers studying mammalian intestinal phosphatase may, therefore, be coincidental.

basis for the determination of the effect of dietary vitamin D on the level of phosphatase in the several tissues. These conditions were adopted in the following study.

Effect of dietary vitamin D on the phosphatase content of plasma and tissues

The possible source of the increased phosphatase of the plasma was investigated by studying the effect of varying amounts of vitamin D in the diet of chicks on the phosphatase content of their bone, kidney, liver, intestinal mucosa and plasma. As in the previous experiments, the several enzyme solutions were pooled extracts obtained from at least twenty chicks in each dietary group. From the data which

Table 1. *The effect of vitamin D in the diet of chicks on the plasma- and tissue-phosphatase activities*

Units of vitamin D/ 100 g. of ration	Phosphatase activity (mg. P)				
	Plasma	Bone	Intestinal mucosa	Kidney	Liver
0	1.65	20.8	47.7	10.2	1.86
10	1.08	14.9	71.6	12.7	1.96
20	0.497	2.96	60.6	12.4	1.80
40	—	—	—	9.7	1.84

The activities of the phosphatases of bone, kidney and liver tissues were found to be maximal in the presence of 0.005M-Mg⁺⁺, the same concentration as that required for maximum activity of chick-plasma phosphatase (Motzok & Wynne, 1950), and half that required for maximum activity of the intestinal phosphatase of the chick.

Optimum substrate concentration. The concentration of substrate chosen for the foregoing studies of the properties of the tissue phosphatases was 0.05M. After the optimum pH and optimum concentration of Mg⁺⁺ had been established for the several phosphatase preparations, it was thought advisable to determine whether or not the concentration of substrate was adequate for maximum rate of hydrolysis under these conditions. With the concentration of enzyme employed and in the presence of H⁺ and Mg⁺⁺ at the optimum concentrations established for 0.05M substrate, maximum activities of the phosphatase of intestinal mucosa and of kidney tissue were obtained with approximately 0.03M-Na glycerophosphate, and for the phosphatases of bone and liver, with 0.05M-glycerophosphate. In no case did increase in the concentration of substrate to 0.06M appreciably change the rate of hydrolysis.

Extensive investigations of the relationship between optimum pH and concentration of substrate, similar to the studies on plasma phosphatase (Motzok & Wynne, 1950), are required in order to evaluate fully the apparent differences between the phosphatases of the various tissues. In the meantime, the above information on the optimum conditions for the activity of the tissue enzymes gives an adequate

are presented in Table 1 it can be concluded that the phosphatase activities of the bone and plasma are markedly influenced by the vitamin D in the diet. A direct and approximately linear relationship was observed when bone-phosphatase activities obtained at different levels of dietary vitamin D were plotted against the corresponding plasma-phosphatase activities. In the case of both enzymes the activities were found to vary inversely with the amount of vitamin D in the diet. The levels of phosphatase activity of kidney and liver extracts were, on the other hand, quite unrelated to the vitamin D content of the diet. Some variation was observed in the activity of the phosphatase of intestinal mucosa, but there appeared to be no correlation of this variation with the level of vitamin D intake.

The present findings support the view that the skeleton is the main source of the increased plasma phosphatase in rachitic chicks. The results also suggest that the level of plasma phosphatase may constitute a suitable criterion for the assay of vitamin D preparations. A detailed study of this possibility has been carried out and is described in another paper (Motzok, 1950).

SUMMARY

1. The pH optimum for the hydrolysis of 0.05M-sodium glycerophosphate was found to be 9.85 for the phosphatase of chick bone, kidney and liver and 9.2 for the phosphatase of the intestinal mucosa. In digestion mixtures containing optimum concen-

trations of H^+ and of substrate, maximum phosphatase activity was obtained at a concentration of 0.005M-magnesium chloride with the enzymes of bone, kidney and liver, and a concentration of 0.01M with the intestinal phosphatase.

2. The phosphatase activities of the plasma and of bone extracts were found to increase markedly and in an approximately parallel manner with diminishing consumption of vitamin D, but those of

the extracts of liver, kidney and intestinal mucosa were not related to the vitamin D intake. These findings support the view of other workers that the increase in plasma phosphatase in rachitic conditions is chiefly the result of 'leakage' of the enzyme from the bones into the blood stream.

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Studies on the Plasma Phosphatase of Normal and Rachitic Chicks

3. THE ASSAY OF ANTIRACHITIC PREPARATIONS BY A METHOD BASED ON THE DETERMINATION OF PLASMA PHOSPHATASE ACTIVITY

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For the determination of vitamin D potency of fish liver oils and other vitamin D-containing substances used in poultry feeding, the Association of Official Agricultural Chemists (1940), and the British Standards Institute (1940) recommend a procedure based on the determination of bone ash. In this method the potency of a vitamin D preparation is determined by comparing the bone-ash values, obtained with the material of unknown potency, with the values obtained with the standard vitamin D preparation. While this is perhaps the most accurate and commonly used chick method, the freeing of the bones from adhering tissue and the extraction of the lipid materials are tedious and time-consuming processes. In comparison with the preparation and

treatment of the bones, the determination of plasma phosphatase activity is a relatively rapid procedure.

As was pointed out previously (Motzok & Wynne, 1950), the increase in the alkaline phosphatase activity of blood has been correlated with the degree of rickets by many investigators. In the case of both animals and man, however, no intensive study of the quantitative relationship between the severity of the rachitic condition and the amount of alkaline phosphatase in the plasma has been reported in the literature. The investigations described in this paper are concerned with the comparison of two methods for the estimation of antirachitic potency of vitamin D preparations: (a) the bone-ash method as outlined