- Herrmann, K. (1958). *Arch. Pharm., Berl.*, **291/63**, 238.
 Herzig, J. & Schönbach, R. (1912). *Mh. Chem.* **33**, 673.
 Horrocks, R. H. & Manning, G. B. (1949). *Lancet*, **256**, 1042.
 Kirby, K. S. & White, T. (1955). *Biochem. J.* **60**, 582.
 Koeppen, B. H. (1961). *Nature, Lond.*, **192**, 360.
 Roux, D. G. (1952). *J. Soc. Leath. Tr. Chem.* **36**, 274.
 Roux, D. G. (1957a). *Nature, Lond.*, **179**, 305.
 Roux, D. G. (1957b). *Nature, Lond.*, **180**, 973.
 Roux, D. G. (1958). *Nature, Lond.*, **181**, 1454.
 Roux, D. G. (1962). *Chem. & Ind.* p. 278.
 Roux, D. G. & Evelyn, S. R. (1958a). *Biochem. J.* **69**, 530.
 Roux, D. G. & Evelyn, S. R. (1958b). *Biochem. J.* **70**, 344.
 Roux, D. G. & Maihs, A. E. (1960). *Biochem. J.* **74**, 44.
 Roux, D. G., Maihs, A. E. & Paulus, E. (1961). *J. Chromat.* **5**, 9.
 Roux, D. G. & Paulus, E. (1960). *Biochem. J.* **77**, 315.
 Roux, D. G. & Paulus, E. (1961a). *Biochem. J.* **78**, 120.
 Roux, D. G. & Paulus, E. (1961b). *Biochem. J.* **80**, 62.
 Roux, D. G. & Paulus, E. (1962a). *Biochem. J.* **82**, 324.
 Roux, D. G. & Paulus, E. (1962b). *Biochem. J.* **84**, 416.
 Shimizu, M., Ohta, G., Yoshikawa, T. & Kasahara, A. (1952). *J. Pharm. Soc. Japan*, **72**, 336.
 Stephen, A. M. (1951). *J. chem. Soc.* p. 646.

Biochem. J. (1963) **87**, 172

Studies on Alkaline Phosphatases

4. INFLUENCE OF DIETARY MINERALS ON pH OPTIMA AND REACTION VELOCITIES OF PHOSPHATASES OF FOWL*

By I. MOTZOK

Department of Nutrition, Ontario Agricultural College, Guelph, Canada

(Received 7 August 1962)

The optimum pH for alkaline phosphatase depends on such factors as the source of enzyme and the type and concentration of substrate, buffers and activators (for references see Motzok & Branion, 1961). Also, the optimum pH of fowl-intestinal phosphatase changes to more alkaline values with increasing age of the birds (Motzok & Branion, 1961). Although the pH optima for the various concentrations of glycerol 2-phosphate were quite constant for the intestinal enzyme of several breeds of chicks at 1 day of age, some differences have been observed among groups of older fowls of approximately the same age but given different experimental and commercial diets. The suggestion that the concentration of specific minerals in the diet caused some of these differences was based on the observation that the optimum pH of intestinal phosphatase was lower in hens given insoluble granite grit than in hens given oyster shell *ad libitum* with a diet containing about 1.2% of calcium (Motzok & Branion, 1961).

The present paper is mainly concerned with the effect of dietary calcium on the pH optima and reaction velocities of the alkaline phosphatase of the intestinal mucosa of fowl. Some observations on the influence of dietary magnesium, manganese and molybdenum on phosphatases of avian tissues are also recorded.

METHODS AND MATERIALS

Methods for the preparation of tissue phosphatases and for the determination of enzyme activity were as described

by Motzok (1950). Except for the studies on pH optima without added Mg^{2+} ions in the reaction mixtures (Figs. 3 and 5), phosphatase activities were determined in the presence of 5 mM- $MgCl_2$ for the enzymes of bone, liver and kidney, and 10 mM- $MgCl_2$ for the enzyme of intestinal mucosa. Columbian Rock, White Rock, Barred Plymouth Rock and Australorp fowls were used. In preliminary work (Expt. 1) hens and 2-month-old cockerels were fed on a commercial diet, with oyster shell and insoluble granite grit being provided to separate groups of birds. In the remainder of the work the experiments were started on 1-day-old chicks, which were randomized into groups of 20 and kept in metal batteries with wire floors in an air-conditioned room maintained at approx. 27°. The experimental diets and water were given *ad libitum*. In studies on the effect of calcium and magnesium a practical type of chick diet was used (Motzok, Arthur & Branion, 1956). This basal diet contained 0.2% of calcium, 0.28% (calc.) of magnesium, 80 p.p.m. of manganese and 0.48% of total phosphorus, of which 0.17% was calculated to be inorganic. Sufficient disodium hydrogen phosphate (commercial grade) was added to the basal diet to raise the concentration of total inorganic phosphorus in the diets to 0.45%. Two dietary concentrations of calcium were employed: calcium carbonate (U.S.P. grade) was added to provide a total calcium content of 0.6% in the low-calcium diet and 1.5% in the high-calcium diet. In experiments with magnesium, 2% of magnesium carbonate (U.S.P. grade) was added. In experiments with supplemental calcium the basal diet constituted 96.6% of the rations and the remaining 3.4% was made up with various amounts of calcium carbonate and finely ground cellulose (Alphacel, obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) (Expt. 2) or with wheat (Expts. 7 and 8). In experiments with added calcium and magnesium the basal diet made up 94.6% of the ration with the remainder provided by the mineral supplements and additional wheat as the other variable component. In studies with manganese

* Part 3: Motzok & Branion (1961).

and molybdenum (Expts. 9 and 10) a diet relatively low in manganese (9–16 p.p.m.) was used (Pepper, Slinger & Motzok, 1952). The molybdenum content of this basal diet was 3 p.p.m. Manganous sulphate pentahydrate (technical grade) and sodium molybdate dihydrate (chemical grade) were employed.

EXPERIMENTAL AND RESULTS

Dietary calcium and magnesium

Effect on optimum pH (Expt. 1). In the initial report on the study in which hens were given oyster shell and granite grit, the pH optima for intestinal phosphatase preparations were given only for 80 mM-disodium glycerol 2-phosphate (Motzok & Branion, 1961). Fig. 1(a) gives the results for all concentrations of substrate which show clearly the difference in pH optima of the enzyme of the two groups of hens. A similar feeding trial was made with Columbian Rock males, commencing at 2 months of age and lasting for 4 months. There was no difference in pH optima (Fig. 1b).

Since no record was made of the amount of oyster shell and grit consumed by the birds and of

their previous calcium intake, it could not be determined whether the difference in pH optima of the enzyme found with hens and not with cockerels was due to an influence of sex on the utilization of calcium or to a difference in the consumption of the supplements. Consequently in the following experiments the supplementary calcium and other test minerals were incorporated into the diets. Fig. 2 gives the results of such an experiment (Expt. 2).

The phosphatase activity of portions of the enzyme preparations of pullets fed on the two diets for 8 weeks in Expt. 2 was destroyed by heating at 80° for 30 min. The optimum pH of 9.45 (with 50 mM-disodium glycerol 2-phosphate and 10 mM-magnesium chloride) of intestinal phosphatase of birds fed on the low-calcium diet was not affected appreciably by the addition of inactivated mucosa of birds fed on the high-calcium diet. Similarly, the optimum of 9.75 (with 50 mM-substrate and 10 mM-magnesium chloride) of the enzyme of birds fed on the high-calcium diet was not altered by the addition of inactivated mucosa of birds fed on the low-calcium diet.

The results of Expts. 1 and 2 indicate that the influence of dietary calcium on optimum pH for the intestinal phosphatase of fowl may manifest itself within a shorter feeding period in females than in males. Therefore only pullets were used in later

Fig. 1. Relationship between the initial concentration of disodium glycerol 2-phosphate, expressed logarithmically, and the optimum pH for the alkaline phosphatase of the intestinal mucosa of fowls (Expt. 1). (a) 2-year-old females; (b) 2-month-old males. Diets: ○, insoluble granite grit; △, crushed oyster shell *ad libitum*; both with a commercial diet for 4 months. Activities were measured in the presence of 10 mM-MgCl₂.

Fig. 2. Relationship between the initial concentration of disodium glycerol 2-phosphate, expressed logarithmically, and the optimum pH for the alkaline phosphatase of the intestinal mucosa of Columbian Rock fowl (Expt. 2). Dietary treatments: ○ and □, 0.6% of calcium; ● and ■, 1.5% of calcium. ○ and ●, Females; □ and ■, males. Period of experiments (from 1 day of age): (a) both sexes, 8 weeks; (b) females, 21 weeks, and males, 35 weeks. Activities were measured in the presence of 10 mM-MgCl₂.

trials. Fig. 3 shows the effect of dietary calcium and magnesium on the optimum pH of the intestinal enzyme (Expt. 3). The activities were determined in the presence and absence of added magnesium chloride in the reaction mixtures.

To verify the effects of dietary calcium and magnesium the experiment of Fig. 3 (a) was repeated. Fig. 4 shows that in Expts. 4 and 5 the

Fig. 3. Relationship between the initial concentration of disodium glycerol 2-phosphate, expressed logarithmically, and the optimum pH for the alkaline phosphatase of the intestinal mucosa of White Rock pullets in the presence of 10 mM-MgCl₂ (continuous lines) and without added MgCl₂ (broken lines) in the reaction mixture (Expt. 3). Dietary treatments: O, 0.6% of calcium; Δ, 1.5% of calcium; □, 0.6% of calcium plus 0.8% of magnesium; ×, 1.5% of calcium plus 0.8% of magnesium; ●, 0.6% of calcium for 6 weeks then changed to 1.5% of calcium; ▲, 1.5% of calcium for 6 weeks then changed to 0.6% of calcium. Feeding periods from 1 day of age: (a) and (b) 6 weeks; (c) 16 weeks; (d) 36 weeks.

differences in pH optima (with magnesium chloride in the reaction mixtures) were similar in all but one respect to those of Fig. 3 (a). The only exception was that in Expt. 4 (Fig. 4a) there was no difference between the pH optima of the enzyme of chicks fed on the low-calcium diet with and without supplemental magnesium.

An attempt was made (Expt. 6) to estimate the time required to produce a clearly defined difference in the pH optima of the intestinal phosphatase of chicks fed on low- and high-calcium diets (Fig. 5). Since Columbian Rock chicks were not then available, Barred Plymouth Rock chicks were employed.

Effect on reaction velocities. Tables 1-3 show the reaction rates at the optimum pH with 80 mM-disodium glycerol 2-phosphate and 10 mM-magnesium chloride of the preparations of intestinal phosphatase obtained from birds used in the experiments with calcium and magnesium reported in the preceding section (Figs. 2-5). Reaction rates are expressed as mg. of inorganic P liberated in 15 min. at 30° by the enzyme in extracts corresponding to 1 g. (dry wt.) of intestinal mucosa.

During studies on the pH optima of intestinal phosphatase of 1-day-old chicks Motzok & Branion (1961) observed that the reaction velocities of enzyme preparations were high, the values being

Fig. 4. Relationship between the initial concentration of disodium glycerol 2-phosphate, expressed logarithmically, and the optimum pH for the alkaline phosphatase of the intestinal mucosa of White Rock pullets in the presence of 10 mM-MgCl₂ in the reaction mixture. Dietary treatments from 1 day to 6 weeks of age: O, 0.6% of calcium; Δ, 1.5% of calcium; □, 0.6% of calcium plus 0.8% of magnesium; ×, 1.5% of calcium plus 0.8% of magnesium. (a) Expt. 4; (b) Expt. 5.

300–500, expressed as in Table 1. Two experiments were made to determine the time required to bring about a marked decrease in the activity of the intestinal enzyme of chicks fed on a high-calcium diet. Two lots of 60 White Rock pullets, each aged 1 day, were fed on diets containing 0.6 and 1.5% of calcium respectively. In Expt. 7, 20 chicks, selected at random from each lot, were killed on the fifth day. The diets of the remaining chicks were interchanged and 20 chicks from each lot were killed on the eleventh day. The diets were reversed again and the remaining chicks were killed on the seventeenth day. Expt. 8 was similar to Expt. 7, except that the diets were interchanged at intervals of 2 days and the chicks were killed at the end of each 2-day feeding period. The results

(reaction velocities at the optimum pH for each enzyme preparation, pH optima and amount of mucosa obtained from the duodenum) are given in Table 4.

It was possible that, when chicks were fed on diets supplemented with different amounts of calcium, the intestinal mucosa contained different amounts of a factor, possibly calcium, which influenced the rate of activity of alkaline phosphatase *in vitro*. However, the addition of inactivated (heated at 80° for 30 min.) mucosa of chicks fed on a high-calcium diet had no effect on the activity of the untreated enzyme preparation of chicks fed on a low-calcium diet (Table 5). Similarly, the activity of the enzyme of chicks fed on a high-calcium diet was not altered by the addition of inactivated mucosa of chicks fed on a low-calcium diet. Further, the amount of phosphate liberated from the substrate by a mixture of the active enzyme preparations was approximately equal to the sum of the phosphate liberated by the two preparations determined separately.

Dietary manganese and molybdenum

Effect on pH optima. In our investigations on manganese and molybdenum metabolism in fowl, the pH optima of alkaline phosphatases of tissues were established before the determination of reaction velocities and other kinetic properties. In Expt. 9 two groups of Columbian Rock cockerels (60 birds per group) were fed on diets containing 9 and 160 p.p.m. of manganese respectively. The optimum pH (for the respective concentrations of disodium glycerol 2-phosphate and 10 mM-magnesium chloride) for intestinal phosphatase was considerably lower in birds given 160 p.p.m. of manganese than in birds given the basal diet

Fig. 5. Relationship between the initial concentration of disodium glycerol 2-phosphate, expressed logarithmically, and the optimum pH for the alkaline phosphatase of the intestinal mucosa of Barred Plymouth Rock pullets in the presence of 10 mM-MgCl₂ (continuous lines) and without added MgCl₂ (broken lines) in the reaction mixture (Expt. 6). Dietary treatments from 1 day of age: (a): ○, 0.6% of calcium and 1.5% of calcium for 2 weeks; ○, 0.6% of calcium; △, 1.5% of calcium; both for 6 weeks. (b): ○, 0.6% of calcium; △, 1.5% of calcium; both for 11 weeks. (c): ●, 0.6% of calcium for 11 weeks then changed to 1.5% of calcium for 10 weeks; ▲, 1.5% of calcium for 11 weeks then changed to 0.6% of calcium for 10 weeks.

Table 1. *Influence of dietary calcium on the activity of the alkaline phosphatase of the intestinal mucosa of the White Rock fowl (Expt. 2)*

Activities were determined at the optimum pH with 80 mM-disodium glycerol 2-phosphate in the presence of 10 mM-MgCl₂ for each enzyme preparation and expressed as mg. of inorganic P liberated in 15 min. at 30° by the enzyme in extracts corresponding to 1 g. (dry wt.) of intestinal mucosa. Further details are given in the text.

Dietary calcium (%)	Sex of birds	Period of experiment (weeks)	Phosphatase activity
0.6	F	8	160
0.6	M	8	113
1.5	F	8	55
1.5	M	8	60
0.6	F	21	51
1.5	F	21	49
0.6	M	35	57
1.5	M	35	48

(9 p.p.m.) (Fig. 6a). In Expt. 10 three groups of Barred Plymouth Rock cockerels (60 birds per group) were fed on a low-manganese (16 p.p.m.) diet containing supplements. There was no difference in pH optima (with disodium glycerol 2-phosphate and 10 mM-magnesium chloride) with diets supplemented with manganese and molybdenum, but these pH optima were lower than those on the unsupplemented diet (Fig. 6b).

The optimum pH of about 10.0 (with 80 mM-disodium glycerol 2-phosphate and 5 mM-magnesium chloride) for the phosphatases of bone and liver of the birds in the two experiments described above was not affected by the additional dietary manganese or molybdenum.

Australorp pullets (40 birds per group) were fed from 1 day to 12 weeks of age on an experimental diet (similar in composition to the high-calcium diet used in the experiments described above) with

and without supplemental molybdenum (500 p.p.m.) (Expt. 11). The pH optima with 80 mM-disodium glycerol 2-phosphate and 10 mM-magnesium chloride were pH 9.55 for the intestinal phosphatase on the diet containing additional molybdenum and pH 9.65 on the unsupplemented diet. On the other hand, maximum activities (with 80 mM-disodium glycerol 2-phosphate and 10 mM-magnesium chloride) of the phosphatases of liver and kidney were at the same pH (about pH 10.0) for the two groups. Supplemental molybdenum also had no effect on the optimum pH 9.96 for plasma phosphatase with 80 mM-glycerol 2-phosphate and 5 mM-magnesium chloride.

Effect on reaction velocities. Table 6 gives the reaction rates (at the optimum pH with 80 mM-disodium glycerol 2-phosphate and 10 mM-magnesium chloride) of the preparations of intestinal phosphatase obtained from chicks in the experi-

Table 2. *Influence of dietary calcium on the activity of the alkaline phosphatase of the intestinal mucosa of pullets*

The determination of reaction rates and expression of activities were as described in Table 1. Further details are given in the text.

Expt. no.	Breed of fowl	Dietary calcium (%)	Period of experiment (weeks)	Phosphatase activity
3	White Rock	0.6	6	136
		1.5	6	31
		0.6 for 6 weeks, changed to 1.5 for 10 weeks	16	37
		1.5 for 6 weeks, changed to 0.6 for 10 weeks	16	43
		0.6 for 6 weeks, changed to 1.5 for 30 weeks	36	46
		1.5 for 6 weeks, changed to 0.6 for 30 weeks	36	68
6	Barred Plymouth Rock	0.6	2	184
		1.5	2	100
		0.6	4	255
		1.5	4	105
		0.6	6	120
		1.5	6	64
		0.6	11	149
		1.5	11	64
		0.6 for 11 weeks, changed to 1.5 for 10 weeks	21	49
		1.5 for 11 weeks, changed to 0.6 for 10 weeks	21	50
		0.6 for 11 weeks, changed to 1.5 for 29 weeks	40	55
		1.5 for 11 weeks, changed to 0.6 for 29 weeks	40	51

Table 3. *Influence of dietary calcium and magnesium on the activity of alkaline phosphatase of the intestinal mucosa of pullets*

The determination of reaction rates and expression of activities were as described in Table 1. Further details are given in the text.

Additions to basal diet*		Phosphatase activity			
Calcium (%)	Magnesium (%)	White Rock			Barred Plymouth Rock
		Expt. 3	Expt. 4	Expt. 5	Expt. 6
0.4	0	136	104	83	120
1.3	0	31	36	45	64
0.4	0.8	44	86	56	66
1.3	0.8	41	50	59	58

* The basal diet contained 0.2% of calcium and 0.28% (calc.) of magnesium.

Table 4. *Influence of dietary calcium on the amount (dry wt.) of mucosa and on the optimum pH and activity of the alkaline phosphatase of intestinal preparations of White Rock pullets*

The determination of reaction rates and expression of activities were as described in Table 1. Further details are given in the text.

Expt. no.	Group no.	Dietary calcium (%)	Period of experiment (days)	Intestinal mucosa* (mg. dry wt. per chick)	Optimum pH	Phosphatase activity
7	1	0.6	5	49	9.46	450
	2	1.5	5	29	9.62	36
	3	1.5 for 5 days, changed to 0.6 for 6 days	11	95	9.50	280
	4	0.6 for 5 days, changed to 1.5 for 6 days	11	54	9.46	41
	5	0.6 for 5 days, changed to 1.5 for 6 days and then to 0.6 for 6 days	17	122	9.46	245
	6	1.5 for 5 days, changed to 0.6 for 6 days and then to 1.5 for 6 days	17	59	9.60	35
8	1	0.6	2	22	9.44	340
	2	1.5	2	18	9.42	190
	3	1.5 for 2 days, changed to 0.6 for 2 days	4	47	9.40	420
	4	0.6 for 2 days, changed to 1.5 for 2 days	4	20	9.46	33
	5	0.6 for 2 days, changed to 1.5 for 2 days and then to 0.6 for 2 days	6	48	9.46	260
	6	1.5 for 2 days, changed to 0.6 for 2 days and then to 1.5 for 2 days	6	32	9.50	39

* Mucosa was obtained from the anterior half of the duodenum.

Table 5. *Alkaline-phosphatase activities determined separately and as mixtures of active and inactivated preparations of the intestinal mucosa of White Rock pullets fed on diets containing different amounts of calcium*

Activities were determined at the optimum pH with 80 mM-disodium glycerol 2-phosphate in the presence of 10 mM-MgCl₂ during a reaction period of 15 min. at 30°. Enzyme preparations were from Expt. 7, groups 1 and 2. The activity of portions of these preparations was destroyed by heating at 80° for 30 min.

Dietary calcium (%)	Enzyme	Amount of mucosa (mg. dry wt./20 ml. of digest)	pH of reaction mixture	Inorganic P liberated (mg./20 ml. of digest)
0.6	Active	0.75	9.46	0.34
1.5	Active	3.75	9.62	0.14
0.6	Active	0.75	9.46	0.34
1.5	Inactive	3.75		
0.6	Inactive	0.75	9.62	0.13
1.5	Active	3.75		
0.6	Active	0.75	9.54	0.45
1.5	Active	3.75		

ments with manganese and molybdenum described above. Values for the enzyme activities were considerably lower in birds fed on low-manganese diets than in birds given these diets with 160 and 400 p.p.m. of manganese (Expts. 9 and 10). The addition of molybdenum to a low-manganese diet (Expt. 10) and a diet containing 80 p.p.m. of manganese (Expt. 11) also resulted in elevated phosphatase activities in the intestinal mucosa.

DISCUSSION

The influence of certain dietary minerals (calcium, magnesium, manganese and molybdenum) on the alkaline phosphatase of intestinal mucosa of

fowl appeared to be on the optimum pH *in vitro* and also on the quantitative activity of the enzyme under optimum conditions. The results of these studies, however, are not sufficient to reveal any definite relationship between the two effects.

Ample evidence was obtained that a marked difference developed between the optimum pH of the intestinal enzyme of chicks fed on the low-calcium diet and that of the enzyme of chicks fed on the high-calcium diet. In White Rock female chicks the difference in optimum pH of the enzyme due to dietary calcium manifested itself in the first 5 days of life (Table 4). The interchanging of the low- and high-calcium diets after the fifth day for a period of 6 days eliminated the difference in

optimum pH, but restoration of the original regime for the following 6 days again produced a difference in pH optima of the enzyme. On the other hand, with Barred Plymouth Rock pullets the difference in optimum pH of the enzyme due to dietary calcium developed at a much slower rate (Fig. 5). Whether this was due to the breed of the fowl or to some other factor(s) is not yet known. Some evidence was obtained (Fig. 2a) that there may be

Fig. 6. Relationship between the initial concentration of disodium glycerol 2-phosphate, expressed logarithmically, and the optimum pH for the alkaline phosphatase of the intestinal mucosa of male fowls in the presence of 10 mM-MgCl₂. Dietary treatments: (a) (Expt. 9): ●, 9 p.p.m. of manganese; ○, 160 p.p.m. of manganese. (b) (Expt. 10): ●, 16 p.p.m. of manganese; ○, 400 p.p.m. of manganese; □, 16 p.p.m. of manganese plus 300 p.p.m. of molybdenum. Breed of fowl: (a) Columbian Rock; (b) Barred Plymouth Rock. The feeding period was from 1 day to 5 weeks of age.

a difference between sexes with respect to the magnitude of the effect of dietary calcium on the optimum pH of intestinal phosphatase of fowl. The utilization or rate of absorption of calcium may have been the common factor that accounted for these variations.

The change in optimum pH of the intestinal enzyme due to the changed calcium content of the diet appeared to take place at a slow rate in older chicks. When the low- and high-calcium diets were interchanged at the end of 6 weeks, the difference in optimum pH was abolished in about 10 weeks (Fig. 3c), and presumably it took at least another 10 weeks of the second dietary phase to produce the difference in optimum pH noted 30 weeks after the diets had been reversed (Fig. 3d). When the concentrations of dietary calcium were not interchanged, a difference in optimum pH was maintained for at least 35 weeks (Fig. 2b).

The calcium content of the diet of chicks also had a remarkable effect on the properties of intestinal phosphatase which govern the extent to which the addition of Mg²⁺ ions *in vitro* changed the optimum pH to lower values (Figs. 3 and 5). When the pH optima were determined with magnesium chloride in the reaction mixtures, maximum activities of the enzyme were at lower pH values in chicks given a low-calcium diet than in chicks given a high-calcium diet. When no magnesium chloride was added to the reaction mixture, the optimum pH of intestinal phosphatase was higher in chicks given the low-calcium diet than in chicks given the high-calcium diet. Thus the change in optimum pH from high to low values when Mg²⁺ ions were added to the reaction mixtures was large for the enzyme of chicks fed on the low-calcium diet but relatively small for the enzyme of birds fed on the high-calcium diet. Indeed, in one trial (Figs. 3a and 3b) there appeared to be no appreciable difference in pH optima for the enzyme

Table 6. Influence of dietary manganese and molybdenum on the activity of the alkaline phosphatase of the intestinal mucosa of chicks

The determination of reaction rates and expression of activities were as described in Table 1. Further details are given in the text.

Expt. no.	Diet	Breed of fowl	Sex	No. of birds	Period of experiment (weeks)	Phosphatase activity
9	Basal*	Columbian Rock	M	60	5	41
	Basal + Mn (160 p.p.m.)	Columbian Rock	M	60	5	80
10	Basal†	Barred Plymouth Rock	M	60	5	80
	Basal + Mn (400 p.p.m.)	Barred Plymouth Rock	M	60	5	107
	Basal + Mo (300 p.p.m.)	Barred Plymouth Rock	M	60	5	131
11	Basal‡	Australorp	F	40	12	48
	Basal + Mo (500 p.p.m.)	Australorp	F	40	12	118

* Basal diet contained 9 p.p.m. of manganese, 1.4% of calcium and 0.90% of phosphorus.

† Basal diet contained 16 p.p.m. of manganese, 1.25% of calcium and 0.95% of phosphorus.

‡ Basal diet contained 80 p.p.m. of manganese, 1.45% of calcium and 0.85% of phosphorus.

of chicks fed on the high-calcium diet when the activities were measured with and without added magnesium chloride in the reaction mixtures. The change in optimum pH of alkaline phosphatase in the presence of a cofactor such as Mg^{2+} ions had been observed previously (Motzok, 1945; Aebi & Abelin, 1948; Sadasivan, 1952; Morton, 1957; Motzok & Branion, 1959), but it was also noted by Aebi & Abelin (1948) that added Mg^{2+} ions had no effect on the optimum pH for the enzyme of rat kidney.

Although calcium and magnesium are antagonists in many biological reactions, the overall effect of increasing the magnesium content of the diets was to diminish (Fig. 4*a*) or completely eliminate (Figs. 3*a* and 4*b*) the marked difference in pH optima of intestinal phosphatase of fowl fed on low- and high-calcium diets (Figs. 3 and 4). Antagonism between calcium and magnesium appeared to manifest itself when the high-calcium diet was supplemented with magnesium; the optimum pH of the enzyme was lower in birds given the high-calcium diet with supplemental magnesium than in birds given the unsupplemented high-calcium diet. A similar effect, however, was not evident when the low-calcium diet was supplemented with magnesium. Indeed, in two of three trials the pH optimum of the enzyme was higher in birds fed on the low-calcium diet with added magnesium than in birds fed on the unsupplemented low-calcium diet. Supplementation of the diets with magnesium, however, did not eliminate the change in optimum pH to lower values when magnesium chloride was added to the reaction mixtures (Figs. 3*a* and 3*b*).

Dietary manganese and molybdenum also influenced the properties of the intestinal enzyme of chicks which govern the pH required for maximum activity. In contrast with dietary calcium, however, the change in optimum pH was to lower values when a low-manganese (9–16 p.p.m.) diet was supplemented with manganese (160–400 p.p.m.) or molybdenum (300 p.p.m.), or when a diet adequate in manganese (80 p.p.m.) was fed with additional molybdenum (500 p.p.m.). Although there was a definite change in optimum pH of intestinal phosphatase of fowl due to dietary manganese and molybdenum, the pH optima for the enzymes of bone and liver were not influenced by dietary manganese and those for the enzymes of bone, liver, kidney and plasma were not altered by dietary molybdenum.

In mammals, the mitotic rate in intestinal mucosa indicates a renewal time of 1–3 days (Leblond & Stevens, 1948; McMin, 1954). The present studies provide indirect evidence that the regeneration of intestinal mucosa occurs in a similar time in chicks. In experiments involving

the interchanging of diets at intervals of 2 and 5–6 days, the amount of mucosa obtained from the duodenum was considerably greater in chicks fed on the low-calcium diet than in chicks given the high-calcium diet in the periods at the end of which the birds were killed (Table 4). The only exception in the two trials occurred in Expt. 8, group 2, where the high dietary calcium has no appreciable effect on the yield of mucosa 2 days after the experiment was commenced. Although no record was made of the daily feed consumption, it is possible that there was a lag in the amount of feed consumed by the chick during the initial stage of the feeding period. A quick regeneration of intestinal mucosa may also account for the rapid changes in the quantitative enzyme activity of chicks when the high- and low-calcium diets were interchanged.

The mechanism of the rapid decrease of phosphatase activity by high dietary calcium, however, remains obscure. No evidence was obtained that the low activity was due to either an increased amount of an inhibitor or a decreased amount of an activator (Table 5). Limited results (Table 3) indicate that high dietary magnesium produced the same results as calcium and that the two minerals were not antagonistic in the process of the rapid decrease in phosphatase activity. The total concentration of these minerals in the diets, rather than their relative amount, may have been the factor involved in this phenomenon.

High dietary manganese and molybdenum also had an effect on the reaction velocities of intestinal phosphatase of chicks. In contrast with high dietary calcium and magnesium, however, manganese and molybdenum maintained the reaction rates of the enzyme at higher levels than that of chicks fed on the unsupplemented diet. In previous work the feeding of molybdenum caused a 50 % increase in the activity of the phosphatase of intestinal mucosa of fowl, but the enzyme activities of the liver and kidney remained about the same (Motzok, Arthur & Branion, 1957).

High dietary calcium caused the phosphatase activity of intestinal mucosa to decline to a low level before an appreciable difference in the optimum pH occurred (Barred Plymouth Rock pullets, Fig. 5*a* and Table 3; White Rock pullets, Table 4). The activity of the intestinal enzyme declined also in birds which received the low-calcium diet, but at a much lower rate than in birds fed on the high-calcium diet. The difference in pH optima due to the calcium content of the diet, however, remained evident for a considerable time after the phosphatase activity of birds fed on the low-calcium diet had eventually diminished to a low level (Figs. 2 and 3, and Tables 1 and 2). Thus the maintenance of characteristics of the enzyme which

governed its optimum pH appeared to depend, at least in part, on a gradient of certain minerals in the intestinal epithelium provided by specific quantities of these minerals in the diet.

Three possibilities could account for the longer time required by dietary calcium and perhaps other minerals to influence optimum pH, in contrast with reaction velocities: (a) a slow alteration of a mechanism in the synthesis of the enzyme; (b) a slow synthesis of an altered enzyme; (c) a slow alteration of the existing residual enzyme after the rapid partial inactivation. The last explanation seems to be the least likely because of the indication that there is a fast regeneration of intestinal mucosa and consequently a rapid renewal of the enzyme.

The present findings emphasize the need for establishing the optimum pH for the phosphatase in each preparation of the intestinal mucosa of the fowl, as perhaps of other species, if maximum enzyme activities are to be obtained. Consideration of all known factors is desirable when phosphatase activity is to be measured under optimum conditions *in vitro*.

SUMMARY

1. In the presence of 10 mM-Mg²⁺ ions the optimum pH of alkaline phosphatase of intestinal mucosa was lower in fowls fed on a low-calcium diet than in those fed on a high-calcium diet. When no Mg²⁺ ions were added to the reaction mixtures, the optimum pH was higher in fowls given a low-calcium diet than in those given a high-calcium diet.

2. Supplemental dietary magnesium reduced the difference in pH optima of intestinal phosphatases of pullets fed on low- and high-calcium diets when the activities were measured with and without added Mg²⁺ ions in the reaction mixtures. High dietary magnesium, however, did not eliminate the change in pH optima to lower values when Mg²⁺ ions were added to the reaction mixture.

3. The phosphatase activity of intestinal mucosa, which was high in chicks at 1 day of age, declined at a much faster rate in birds given a high-calcium diet than in birds given a low-calcium diet. High dietary magnesium also caused a rapid decrease in the enzyme activity.

4. High dietary calcium caused intestinal phosphatase activity to decline to a low level before an appreciable difference developed in the optimum pH for the enzymes of chicks given a low-calcium

diet and those given a high-calcium diet; the difference in optimum pH, due to the amount of calcium in the diets, persisted after the enzyme activity of birds fed on a low-calcium diet had also declined to a low level.

5. The interchanging of the low- and high-calcium diets at intervals of 2 and 5-6 days resulted in reversals of the quantitative activities of intestinal phosphatase and the amounts of mucosa obtained from the duodenum of chicks.

6. The quantitative activity of intestinal phosphatase of chicks fed on a low-calcium diet was not influenced by the addition of inactivated (heated at 80° for 30 min.) enzyme preparation of chicks fed on a high-calcium diet, and the activity of the enzyme preparation of chicks fed on a high-calcium diet was not altered by inactivated mucosa of chicks fed on a low-calcium diet. The reaction velocity of a mixture of the active preparations from the two dietary groups of chicks was approximately equal to the sum of the activities of the two preparations measured separately.

7. In the presence of 10 mM-Mg²⁺ ions the optimum pH of intestinal phosphatase was lower in birds given diets supplemented with manganese or molybdenum than in birds given the unsupplemented diets.

8. Dietary supplements of manganese or molybdenum caused an increase in the phosphatase activity of intestinal mucosa.

The author is grateful to Miss G. Ritcey for valuable technical assistance and to Mr G. Amos, Mr A. Richardson and Mr E. Emslie for their help in the preparation of diets and in maintaining the fowls. He also thanks Dr H. D. Branion for his interest in this work and Dr D. C. Hill for useful discussions and constructive criticism of the manuscript.

REFERENCES

- Aebi, H. & Abelin, I. (1948). *Helv. chim. acta*, **31**, 1943.
Leblond, C. P. & Stevens, C. E. (1948). *Anat. Rec.* **100**, 357.
McMinn, R. M. H. (1954). *J. Anat. Lond.*, **88**, 527.
Morton, R. K. (1957). *Biochem. J.* **65**, 674.
Motzok, I. (1945). Ph.D. Thesis: University of Toronto.
Motzok, I. (1950). *Biochem. J.* **47**, 193.
Motzok, I., Arthur, D. & Branion, H. D. (1956). *Poultry Sci.* **35**, 627.
Motzok, I., Arthur, D. & Branion, H. D. (1957). *Poultry Sci.* **36**, 1144.
Motzok, I. & Branion, H. D. (1959). *Biochem. J.* **72**, 177.
Motzok, I. & Branion, H. D. (1961). *Biochem. J.* **80**, 5.
Pepper, W. F., Slinger, S. J. & Motzok, I. (1952). *Poultry Sci.* **31**, 1054.
Sadashivan, V. (1952). *Nature, Lond.*, **169**, 418.