Tissue Components of the Domestic Fowl

4. PLASMA-ALKALINE-PHOSPHATASE ACTIVITY*

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The alkaline-phosphatase activity of the plasma (or serum) of the domestic fowl has been the subject of a number of investigations by various methods of assay (Auchinachie & Emslie, 1934; Common, 1934, 1936; Peterson & Parrish, 1939*a, b*; Wiese, Johnson, Elvehjem, Hart & Halpin, 1939; Motzok, 1950*a, b*, 1959; Stutts, Briles & Kunkel, 1957). In general, single measurements were made on individual birds although occasionally subsequent measurements were made on the same animal; however, Common (1936) records a number of experiments where white Wyandotte pullets and cockerels were examined at approximately monthly intervals. There is agreement that the plasma activities in cocks and in immature birds show less variability between individuals than do the activities in hens and that no significant differences could be demonstrated between hens when 'in' or 'out of lay'. There are no records of measurements more frequent than monthly on individual birds during a lengthy period of observation.

The plasma enzyme is assumed to have its origin in the bones, and to be associated with osteoblastic activity (Hall & King, 1930-31; Franseen, Simmons & McLean, 1939; Woodard & Higinbotham, 1941; Cade, MacLagan & Townsend, 1940; and histological observations in this Laboratory). During the investigation at this Centre of an outbreak of 'cage-layer fatigue' (cf. Couch, 1955; Francis, 1957) it became necessary to study the plasma-alkaline-phosphatase activity in healthy birds, since Bell, Siller & Campbell (1959) had found that the syndrome in question involves a bone dystrophy.

This paper describes a simple spectrophotometric micromethod to measure the enzymic hydrolysis of 4-nitrophenyl phosphate and includes observations made at frequent intervals over 6 months on a small group of hens kept in the same environment.

METHODS

The birds examined were brown Leghorns of the flock maintained on the standard diet (Bell, 1957). Blood samples were taken from the wing vein into a heparinized syringe and the plasma was separated as soon as possible

after bleeding. In agreement with Motzok & Wynne (1950), the alkaline-phosphatase activity of fowl plasma against both 4-nitrophenyl phosphate and glycerol 2-phosphate was not found to alter on keeping the plasma for 2 weeks in the refrigerator.

Alkaline-phosphatase activity of plasma

4-Nitrophenyl phosphate was first used by Ohmori (1937) as a substrate in visual colorimetric measurements of enzymic activity; later, Bessey, Lowry & Brock (1946) used this substrate in a micro-assay on human blood which employed but 5 μ l. of plasma. The simplified method described below owes much to these authors and to the work of J. D. Bell (1955).

Reagents. The buffer (Delory & King, 1945) consisted of 0.1 M-Na₂CO₃-0.1 M-NaHCO₃ (7:3), pH 10.08 at 37°. The substrate consisted of 0.066 M-disodium 4-nitrophenyl-phosphate (L. Light and Co. Ltd.) recrystallized according to Ohmori (1937). Free nitrophenol was removed from the solution by threefold extraction with water-saturated butan-1-ol followed by extraction with moist ether; dissolved ether was removed by a stream of moist air. Although the solution was stored in the refrigerator some slight hydrolysis of the ester took place but this was not of consequence since the blanks contained substrate equal to that used in the digests. The standards were prepared from a stock aqueous solution of purified 4-nitrophenol (200 μ g./ml.) by dilution with 0.05 M-NaOH to concentrations (μ g./ml.) of 0.25, 0.50, 1.25, 2.50, 5.0, 10.0. With a 1 cm. light path these solutions gave a linear graph when measured at 397 m μ in the Unicam SP. 500 spectrophotometer. (Other authors have recorded their measurements at 400 m μ ; on our instrument a definite maximum was found at 397 m μ .)

Activity measurements. Owing to the wide variations between plasma samples in colour and turbidity it was found necessary to run a plasma blank with each digest. In this way considerable colours in the sample, even visible amounts of haemoglobin, are compensated for.

Buffer (1 ml.) was placed in suitable vessels, e.g. test tubes 15 cm. $\times$ 1.6 cm., followed by plasma (0.05 ml.); after mixing at room temperature (about 15°) substrate (0.1 ml.) was quickly added and mixed with the contents of each vessel, all of which were then kept at 37° for 60 min. (although the body temperature of the fowl is 41.5°, the former temperature was used as it is customary for laboratory incubation). At the end of the incubation period the mixture in each vessel was diluted with a measured volume of 0.05 M-NaOH; according to the enzymic activity of the plasma the amount of diluent added may vary between 3 and 25 ml. in order to obtain a final concentration of 4-nitrophenol of the order of 2-7 μ g./ml. The method has thus considerable flexibility.

* Part 3: Bell, McIndoe & Gross (1959).

Table 1. *Comparison of Bodansky, King-Armstrong and 4-nitrophenol assays of alkaline-phosphatase activity in plasmas of hens*

Ratios are calculated on the basis of the inorganic phosphate liberated from each substrate in 60 min. at 37° in 0.1M-Na₂CO₃-NaHCO₃ buffer, pH 10.08, by 100 ml. of plasma. Activities are expressed in the conventional 'units'.

Date of assay	No. of hen	Activities			Ratios		
		Bodansky (B)	King-Armstrong (KA)	4-Nitrophenol (NP)	NP/B	NP/KA	KA/B
19. ii. 59	1	13.6	149	118.0	1.9	0.54	3.6
	8	3.0	31.8	27.0	2.0	0.57	3.5
23. ii. 59	8	—	20.3	18.0	—	0.61	—
26. ii. 59	1	3.2	37.4	31.4	2.2	0.57	3.8
	2	2.9	30.7	25.8	2.0	0.57	3.5
	3	1.8	15.4	15.4	1.9	0.67	2.8
	4	3.4	31.3	28.9	1.9	0.63	3.4
	5	—	36.7	35.7	—	0.66	—
	6	—	27.4	24.0	—	0.59	—
3. iii. 59	1	—	32.0	25.8	—	0.55	—
	2	—	50.0	53.5	—	0.72	—
	3	—	29.5	31.4	—	0.71	—
	4	—	26.0	22.0	—	0.59	—
	5	—	36.5	29.5	—	0.55	—
	6	—	40.5	36.7	—	0.64	—
	8	—	22.5	20.0	—	0.63	—
				Means	1.99	0.61	3.4

A blank was set up for each plasma sample and incubated with the digests; the blank contained buffer (1 ml.) and plasma (0.05 ml.). At the end of the incubation, 0.05M-NaOH was added in amount equal to that added to the digest. After mixing, substrate (0.1 ml.) was added and the whole mixed. Each diluted digest was measured at 397 m μ against its blank and the 4-nitrophenol content obtained from the standard curve.

Excellent agreement between duplicates was obtained; furthermore, if the solutions were kept in the refrigerator no change in the difference between digest and blank could be found after 24 hr.

Activity. This is expressed as mg. of 4-nitrophenol liberated by 100 ml. of plasma in 60 min. at 37° with the buffer and substrate mentioned above.

RESULTS

A comparison of three methods of assay of plasma-alkaline-phosphatase activity. The 4-nitrophenyl phosphate method was compared with assays by the methods of Bodansky (after Hawk, Oser & Summerson, 1947) and King & Armstrong (1934); the latter estimations were done independently by Mr T. P. Bird. In all estimations the same buffer and period of incubation were used; the results are given in Table 1. Except for this experiment all measurements described below were made by the nitrophenyl phosphate method.

As stated in Table 1, the ratios between the activities measured by the three methods are assessed from the amounts of phosphate liberated from three different esters under the same condi-

tions of incubation; the conventional concentrations of glycerol 2-phosphate and phenyl phosphate were used. Although it is well known that it is not easy to obtain close agreement between measurements of alkaline-phosphatase activities against different substrates, each of the three ratios (nitrophenyl phosphate method/Bodansky method, nitrophenyl phosphate method/King-Armstrong method and King-Armstrong method/Bodansky method) varies between reasonably narrow limits. These variations may be due either to errors of manipulation or to the alkaline phosphatase of the plasma consisting of a mixed and variable population of enzymes having different activities against the different substrates. The ratios are sufficiently constant to enable the orders of the results of others, by any of the three methods, to be assessed in terms of either the Bodansky, the King-Armstrong or the nitrophenyl phosphate method.

A survey of plasma-alkaline-phosphatase activity in six hens in different physiological states. Six hens (nos. 1-6) chosen for high laying rate and in their second year of production, were examined at frequent intervals from 21 November 1958 (when they were in moult) until 29 May 1959; in all, 75 measurements were made on each of five birds and 58 on the sixth. The birds were caged in a well-ventilated room and, when in lay, received light for 14 hr./24 hr.; the temperature of the room was not controlled, but the birds were not subjected to the vagaries of temperature experienced in the general animal house. The findings are given in Tables 2 and 3.

Table 2 is an attempt to represent, in a condensed form, a large number of observations. It is clear that the activity of the plasma enzyme in one hen can be very different from that in another; it therefore is illogical to attempt to derive mean figures for the groups in the different physiological states. During the moulting and post-moulting non-laying periods each bird maintained a relatively steady level of activity, as indicated from the s.d. values. The first 2 months of laying were marked by large fluctuations in the plasma-alkaline-phosphatase activity; the fluctuation in activity decreased very markedly during the subsequent months in four birds whereas the remaining two continued to show rises and falls of the same order as they did when coming into lay. In all these hens, the lowest levels of enzymic activity were of much the same order whatever the physio-

logical state. Since the metabolism of the heavily laying hen is in a state of constant flux it is surprising that any agreement at the lower levels of enzymic activity can be observed at all without taking precautions to obtain the plasma at identical times during the laying cycle.

The results expressed in Table 3 amplify those of Table 2. Hens nos. 1, 2 and 6 appear relatively homeostatic whereas nos. 3 and 4 show wide day-to-day fluctuation.

Cocks and immature birds. Six adult cocks were observed for 5 successive days (Table 4) and a few single measurements were made on immature birds of both sexes and some repetitions on the females (Table 5).

The cocks, which were kept in the same pen, showed quite characteristic individual levels with little fluctuation; the values found for plasma-

Table 2. *Alkaline-phosphatase activity in plasma of six hens in three physiological states*

Condition	Hen no.	Period covered by observations (dates inclusive)	No. of observations	Plasma-phosphatase activity				Eggs laid
				Highest	Lowest	Mean	s.d.	
Moulting and refeathering	1	21. xi.-8. xii. 58	8	97	39	64	18.2	—
	2	21. xi.-1. xii. 58	5	56	29	43	10.9	—
	3	21. xi.-18. xii. 58	11	60	26	42	12.0	—
	4	21. xi.-12. xii. 58	10	36	18	28	6.4	—
	5	21. xi.-10. xii. 58	9	56	31	41	9.4	—
	6	21. xi.-28. xi. 58	4	83	56	71	13.4	—
Non-laying (post-moult)	1	10. xii. 58-27. i. 59	16	112	43	82	19.1	—
	2	3. xii. 58-26. xii. 58	8	60	32	53	12.5	—
	3	22. xii. 58-30. i. 59	16	68	24	39	11.3	—
	4	18. xii. 58-2. i. 59	6	92	27	52	22.0	—
	5	12. xii. 58-29. xii. 58	5	68	39	53	10.5	—
	6	12. xii. 58-19. i. 59	13	116	43	64	18.6	—
Laying, first 28 days	1	27. i. 59-23. ii. 59	8	300	42	125	87.1	20
	2	28. xii. 58-24. i. 59	10	135	22	49	32.6	12
	3	2. ii. 59-1. iii. 59	7	186	15	73	68.0	16
	4	5. i. 59-1. ii. 59	9	166	29	75	55.6	16
	5	30. xii. 58-26. i. 59	11	175	31	80	47.7	18
	6	21. i. 59-17. ii. 59	8	184	37	99	50.5	18
Second 28 days	1	24. ii.-23. iii.	8	133	26	55	36.0	24
	2	25. i.-21. ii.	7	160	45	102	43.4	17
	3	2. iii.-29. iii.	8	93	18	44	25.7	22
	4	2. ii.-1. iii.	7	280	29	124	85.5	16
	5	27. i.-23. ii.	7	166	41	100	46.5	7*
	6	18. ii.-17. iii.	8	109	24	62	28.7	20
Third 28 days	1	24. iii.-20. iv.	15	61	28	40	10.2	23
	2	22. ii.-21. iii.	8	55	18	40	13.5	16
	3	30. iii.-26. iv.	15	55	17	35	8.4	22
	4	2. iii.-29. iii.	8	152	25	77	55.2	20
	5	24. ii.-23. iii.	8	87	30	47	19.3	8*
	6	18. iii.-14. iv.	14	157	26	58	37.4	20
Fourth 28 days	1	21. iv.-18. v.	11	58	20	40	12.9	23
	2	22. iii.-18. iv.	15	69	20	37	16.4	16
	3	26. iv.-24. v.	13	145	28	78	33.3	18
	4	30. iii.-26. iv.	18	210	29	86	56.5	22
	5	24. iii.-22. iv.	15	72	21	47	14.1	13*
	6	15. iv.-12. v.	13	72	25	46	13.2	21
Fifth 28 days	2	19. iv.-16. v.	11	55	30	42	8.82	22
	4	27. iv.-24. v.	13	290	65	130	60.30	22

* This hen was killed on 22. iv. 59 for post-mortem examination. It had been 'laying internally', i.e. the yolks had passed into the peritoneal cavity and therefore no shells had been formed on many potential eggs.

alkaline-phosphatase activity were of the same order as the lowest levels of the non-laying and laying hens (Table 2).

The immature birds (5-6 weeks old) showed high values, as might be expected in rapidly growing animals. At 8½-10 weeks, however, it will be seen that many of both sexes had plasma enzymic activities more of the order of those found in adult hens, although rapid growth of these birds continues for some weeks at apparently the same rate as the 5-6-week-old animals.

Table 3. *Groups of daily measurements of plasma-alkaline-phosphatase activity on five laying hens in spring 1959*

Periods when measurements were made; A, 1-3. iv.; B, 6-10. iv.; C, 13-17. iv.; D, 27. iv.-1. v.; E, 4-8. v.; F, 25-29. v.

Period	Plasma-alkaline-phosphatase activity				
	Hen 1	Hen 2	Hen 3	Hen 4	Hen 6
A	33	20	17	29	42
	32	28	40	38	85
	54	29	44	75	157
B	42	40	40	58	46
	34	23	20	32	45
	36	27	23	41	47
	36	36	37	49	41
C	41	41	26	120	62
	28	31	41	41	31
	22	28	38	81	28
	27	45	32	120	51
	20	63	40	210	44
D	38	69	40	135	34
	28	55	55	74	37
	22	38	45	65	37
	27	34	47	101	25
	20	34	94	153	37
E	38	43	145	290	63
	48	44	71	82	46
	47	30	62	88	50
	44	47	97	105	60
	58	46	72	125	53
F	57	52	49	130	72
	56	116	43	195	41*
	75	92	28	220	57
	58	85	33	135	43
	61	128	59	168	38
	77	117	58	115	40

* Hen no. 6 was out of lay between 12. v. and 30. v.

Table 4. *Daily plasma-alkaline-phosphatase activity of six adult cocks*

Cock	Consecutive days					Mean
	1	2	3	4	5	
A	37	23	20	24	23	25
B	57	42	41	42	30	40
C	65	42	41	48	49	59
D	81	62	61	71	79	71
E	47	33	33	39	38	38
F	32	20	23	26	30	26

DISCUSSION

Throughout the following discussion it must be borne in mind that the plasma alkaline phosphatase of the healthy fowl is understood to originate in the bones, and particularly in the osteoblasts; the enzymic activity of the plasma therefore reflects the activity of a mechanism for skeletal calcification (cf. Hall & King, 1930-31; Auchinachie & Emslie, 1934; Common, 1934, 1936; Motzok, 1950*a, b*). Egg-shell calcification, on the other hand, is mediated by carbonic anhydrase in the shell gland (Common, 1941; Gutowska & Mitchell, 1945) and therefore is not directly dependent on any mechanism in which alkaline phosphatase is concerned. Indirectly, however, a high proportion of the shell requirements have to be met from skeletal sources; Driggers & Comar (1949) and Jowsey, Berlie, Spinks & O'Neil (1956) have demonstrated, with ⁴⁵Ca, that food in the gut supplies rather less than half of shell calcium. The bones have long been known to be a source of shell calcium; in the immediate pre-laying weeks, increased mineralization of the skeleton can be observed; in eight of ten hens observed in the present series a definite peak in plasma-alkaline-phosphatase activity was noted to precede the onset of lay.

There must be a considerable turnover of skeletal calcium (cf. Winget & Smith, 1958; Winget, Smith & Hoover, 1958) in the laying hen and the very active recalcification concerned would be expected to cause an increase in plasma phosphatase, as indeed was observed during the early months after the onset of lay (Table 2). Despite the maintenance of a high rate of production, in three hens (nos. 1, 2 and 6) the level of enzymic activity in the plasma became less and less variable as time went on. In nos. 3 and 4, on the other hand, variability remained high throughout the months of observation (Table 3). The behaviour of no. 5, which was found to be 'laying internally', is of some interest as this hen produced relatively few shelled eggs although it was known to have active ovaries, from the continued presence in its plasma of the characteristic 'lipophosphoprotein complex' (McIndoe, 1957, 1959). The fall in variability in plasma-alkaline-phosphatase activity in this bird resembles that of nos. 1, 2 and 6.

An explanation of the differences between the behaviour of the different birds is not obvious, but several suggestions may be offered. Homeostasis with respect to the mechanisms affecting calcium in circulation between the gut, the skeleton and the oviduct may have become established in nos. 1, 2 and 6, whereas in nos. 3 and 4 a periodical and repetitive osteoblastosis alone may have caused the continued violent fluctuations in plasma

Table 5. *Plasma-alkaline-phosphatase activity in immature fowls*

Figures in parentheses refer to assays on the same bird made 4 days later.

Age (weeks)	No. of birds	Sex	Plasma activity
5-6	11	M.	404, (406); 442, (442); 362, (402); 292, (298); 276, (202); 432 (484); 290; 480; 510; 340; 330
	11	F.	260 (210); 360, (380); 250, (280); 280, (280); 330 (350); 180 (160); 260; 520; 350; 150; 340
8½-10	9	M.	280; 99; 120; 320; 170; 85; 157; 210; 110
	8	F.	99; 85; 170; 160; 150; 126; 510; 120

Fig. 1. Diagrammatic representation of the fluctuations in the plasma-alkaline-phosphatase activity of six laying hens during four to five successive 28-day periods. The hatched portions relate to the 28-day period immediately before onset of lay. (For actual levels of activity see Table 2.)

phosphatase. If, as in mammals, plasma alkaline phosphatase is eliminated via the bile, a flexibility in the excretory mechanism of the liver may have developed in nos. 1, 2 and 6 but not in nos. 3 and 4. A third alternative lies in the possibility that improved intestinal absorption, or a decreased excretion, of calcium develops in some birds but not in others, thus sparing transfer of skeletal calcium to the shell.

That individual birds can possess differing biochemical features is illustrated by Tables 2 and 3. Such variabilities in alkaline-phosphatase activity are to be found that an isolated measurement will seldom provide information of value, especially in the laying hen. Common (1936) found, in white Wyandotte pullets, distinct evidence of considerable temporary fluctuations after the onset of lay although his measurements were made at approximately monthly intervals. One of Common's birds showed a remarkably high level of activity comparable with that found by Bell, Siller & Campbell

(1959) in cage-layer fatigue. Daily fluctuations can be considerable, as seen from Table 3 (nos. 3 and 4). Bloom, Domm, Nalbandov & Bloom (1958) have shown that, in hens with a good laying record, the medullary bone undergoes sequences of resorption and recalcification concomitant with the formation of the shell. During early stages of shell calcification the medullary bone is rich in both osteoblasts and osteoclasts; as calcification becomes advanced osteoblasts become scarce and osteoclasts is pronounced. During the interval between laying and the next ovulation the numbers of both osteoblasts and osteoclasts in the medullary bone are very variable. If indeed the osteoblasts are the source of plasma alkaline phosphatase, variations in the activity of these cells in the medullary bone may account for some of the fluctuations noted in the present series of experiments.

For comparison purposes, a few daily observations were made on adult cocks (Table 4). Although the activity remained within narrow limits for an individual bird, those limits show a scatter between 25 and 71 units; immature birds of 5-6 weeks (Table 5) have very high activity, as might be expected in growing animals. The values found in a few young birds of 8½-10 weeks show irregularity and some values approach the adult level; Dr J. G. Campbell (unpublished work) has obtained other evidence of rapid fluctuations in the oestrogen-androgen balance of the 6-12-week-old bird. Exogenous sex hormones administered to such immature birds have not yet been shown to influence the plasma-alkaline-phosphatase activity.

It is concluded that egg-shell formation in heavy layers is accompanied by elevated osteoblastic activity, as evinced by an increased fluctuation in the level of the plasma-alkaline-phosphatase activity during the first month after the onset of lay (Fig. 1). Birds which continue to lay eggs with normal shells, at a high rate, may show persistent fluctuations in plasma phosphatase, whereas others become, in some way not yet understood, stabilized with respect to the level of enzyme in their plasma. Isolated analyses on individual birds can easily lead to false conclusions.

SUMMARY

1. A simple and flexible method is described for assessing plasma-alkaline-phosphatase activity against 4-nitrophenyl phosphate. It has been compared with the Bodansky and the King-Armstrong procedures.

2. Both adult non-laying hens and cocks show enzymic activity of the same order. The plasmas of immature birds (5-6 weeks old) of both sexes have alkaline-phosphatase levels up to ten times those found in non-laying adults. At 8½-10 weeks of age great irregularities may be found in that the plasma-alkaline-phosphatase activity of an individual may have dropped to a level near that of the adults, although these birds continue to grow at the same rate as those of 5-6 weeks.

3. In the hen, the average level of plasma enzymic activity is increased by about 50 % upon the bird coming into lay, when wide fluctuations become apparent. The lowest levels in the laying hen are, however, of the same order as those found in the non-laying bird and in the cock.

4. In some hens a fairly steady level of activity with small fluctuations is attained after the first 2 months of intensive egg production. Other birds, however, continue to show very large fluctuations in alkaline-phosphatase activity with no apparent tendency to the establishment of homeostasis with respect to this enzyme.

5. The massive temporary increases in plasma phosphatase shown by the laying hen are believed to reflect a stimulation of the osteoblastic recalcification process called into action by the drain on skeletal calcium required for egg shells.

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