BLOOD CALCIUM STUDIES IN THE FOWL

by

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INTRODUCTION

That there is an increase in the blood calcium of birds at the onset of egg production apparently was first observed by Riddle and Reinhart (1926), who worked upon pigeons. The next year Hughes, Titus, and Smits (1927), and later Macowan (1932) demonstrated that a similar phenomenon occurred in chickens. They found that immature fowls, non-laying hens, cocks, and capons usually have from 12 to 18 mg. of calcium per 100 cc. of blood serum or plasma, and that actively laying hens have levels ranging from 17 to 35 mg. of calcium.

The mechanism responsible for this rapid increase in blood calcium has not been adequately explained. Many theories of it have been advanced but none proven. It is probable that the diversity of opinions expressed at the different laboratories might be attributed to a lack of appreciation of the large number of factors, environmental and otherwise, which might influence the level of calcium in the blood of birds.

Many of the experiments have not been adequately controlled. The fact that blood calcium can vary over a wide range in the same bird emphasizes the need for positive[1] controls. Calcium determinations on the same bird before and after treatment may be quite misleading.

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[1] Positive controls are birds which have been paired with experimental birds and given the same set of conditions except for injections of the test substance.

Diurnal fluctuations due to environment or to egg cycle may be important as shown by Knowles, Hart, and Halpin (1935), who found a variation in the level of blood calcium in laying hens from one oviposition to another. The maximum and minimum were not constant even for the same hen; the period of low level corresponded to the time taken for shell deposition, and the period of high level corresponded to the absence of a fully formed egg in the shell gland. However, Deobold, Lease, Hart, and Halpin (1936) found that in individual laying hens the blood calcium content remained quite constant. This is compatible with the findings of Feinberg, Hughes, and Scott (1937) who showed that the calcium level was relatively constant during the 26-hour period of a single egg cycle.

That the diet may be an important factor was shown by Morgan and Garrison (1930) who, working with dogs, showed that animals reared on a vitamin D deficient diet responded but little to injections of a potent parathyroid extract; the serum calcium was raised only slightly. Similar dogs given an abundance of vitamin D showed large increases in blood serum calcium when given injections of parathyroid extract. There is also the possibility that variability in the amount of calcium in the gut of the bird would cause blood calcium levels to vary.

Temperature was shown by Conrad (1939) to exert a marked influence upon blood calcium of laying hens. He reported that an increase in temperature from 70°F. to over 90°F. caused decreases of from 20 to 30 per cent in the blood calcium level. Other important factors which

might contribute to the present state of confusion are source, dosage, and method of injecting extracts. There is also the possibility that frequency of handling and frequency of bleeding might cause fluctuations. The sex and age of birds are important, as has been demonstrated by many workers. The method of analysis used might also be a factor which would cause workers to report conflicting results.

The causal factors associated with the fluctuation in blood calcium in the fowl have been the subject of investigation at the Kansas Agricultural Experiment Station, dating from the work of Hughes, Titus, and Smits (1927). An endeavor has been made in this problem to study the effect of parathyroid and estrogenic preparations upon the blood calcium levels in the fowl.

REVIEW OF LITERATURE

The responses following injections of parathyroid extracts have not been consistent in the fowl. Collip (1931), as reported by Hutt and Boyd (1935), was unable to demonstrate any effect of the hormone in non-laying hens. Macowan (1932) reported similar results with molting hens when injections as large as 1 cc. were given; however, she found that the serum calcium was distinctly raised in from 15 to 19 hours after a single injection of .5 cc. of parathormone into pullets. The degree of sexual development of the pullets was not given.

A suggestion that the increase in blood calcium of the laying hen might be due to the activity of the parathyroid gland was demonstrated

by the work of Hutt and Boyd (1935). They reported the case of a pullet weighing 2.2 kg. which, after laying 11 eggs, was afflicted with tetany and paresis, and was found to have only 9.47 mg. of calcium per 100 cc. of blood plasma. Liberal doses of calcium gluconate had no effect, and tetany was succeeded by coma. Treatment with .5 cc. of parathormone (Lilly) given intramuscularly on the fifth day resulted in complete recovery. These authors felt that the effectiveness of parathormone, when injections of calcium had failed, suggested that its function is not so much to raise the level of total blood calcium as to convert some of the calcium into a form, or forms, indispensable to the bird.

Knowles, Hart, and Halpin (1935) were able to demonstrate an increase in blood calcium of immature pullets and non-laying hens when 1 to 3 cc. of parathormone were given. Positive controls were not used. The peak of the rise came from 2 to 10 hours after injections, the rise being rapid and sudden, as is illustrated in their Table 7. Although they do not so state, it is evident from this table that similar injections failed to produce a significant response in capons or cocks. However, Deobold, Lease, Hart, and Halpin (1936) reported that subcutaneous injections of 1 cc. of parathormone (Lilly) administered twice daily into a laying hen which had been starved of calcium exerted no effect on blood calcium level.

Altmann (1938), injecting from 2 to 4 cc. of parathyroid extract (Squibbs and Sons) intramuscularly over a 2-day period into immature

pullets, reported an increase of 47.17 per cent in blood calcium. It is worthy of note, however, that the positive controls increased 20.46 per cent. The same worker made simultaneous injections (intramuscularly) of 3 cc. of anterior pituitary extract and 2 to 3 cc. of parathyroid extract into each of two 150-day old pullets over a 3-day period. Positive controls were not used. The blood calcium dropped 26 per cent by the third day, but had returned to normal by the tenth day. Injections of 5 cc. of follicle stimulating hormone (F.S.H.) and 200 to 400 units of parathyroid extract were made into two 85-day old pullets over a period of five days. Similar injections of parathyroid extract alone were made. A 44 per cent rise in blood calcium was obtained where both extracts were used, and a 47 per cent increase where only the parathyroid extract was used. It was concluded that the F.S.H. had no effect.

Macowan (1932) showed that the rise in blood calcium was coincident with ovarian activity and reached a maximum level when the individual follicles weighed from 10 to 30 g. The blood calcium level then fell only slightly as the ovum developed until the egg was laid.

Riddle and Dotti (1936) reported that sufficient and prolonged dosage of certain female sex hormones, notably theelin and to a lesser extent dihydrotheelin and theelol progesteron, increased the serum calcium in normal castrate hypophysectomized, thyroidectomized pigeons and in normal doves and fowl. The response of the rat and the dog was not so distinct.

Levin and Smith (1938) studied the blood calcium in rats, rabbits, and monkeys before and after the administration of estrogenic material. They seriously doubted whether estrin actually has a physiologically significant effect on mammalian blood calcium. They felt that in those cases where huge doses of estrin did have a statistically significant effect, the increase was only transitory since serum calcium returned to normal values if treatment was continued. Altmann and Hutt (1938), working with chickens, noted that blood calcium levels tend to return to the original level while injections were still being made. They advanced the hypothesis that the formation of antihormones or antibodies counteracted the effects of the agents injected. It seems that if this were the case, these antihormones or antibodies would be formed when the hen's blood calcium rose at time of egg production.

Marlow and Koch (1937) prepared a non-crystalline purified estrogenic product from pregnancy urine, but were unable to produce a significant and consistent effect on the blood calcium level in fowls. A male hormone concentrate prepared from bull testis tissue also produced no change in the blood calcium level of the fowl.

Zondek and Marx (1939) reported blood calcium values as high as 55.3 mg. per cent in cocks which had been treated with oestrodiolbenzoate for a considerable length of time. Neither the age of the cocks nor the mode of administering the preparation were mentioned.

Riddle and Dotti (1936) reported that the administration of theelin (progynon) in doses of 200 R.U. daily for seven days into

hypophysectomized pigeons produced a rise from 9.4 mg. of calcium per 100 cc. of blood to 22.3 mg. of calcium. The same workers, Riddle and Dotti (1938), stated that sex hormones show extreme differences in their capacity to increase the serum calcium. The female sex hormones theelin and dihydrotheelin benzoate markedly increased the serum calcium in both sexes of mature and immature fowl. Most pronounced increases were shown after 2,000 R.U. had been injected daily for a period of seven days.

Pfeiffer and Gardner (1938) reported that injections of estrogen into pigeons was followed by a rise in serum calcium level and hypercalcification of the bones. They reported that serum calcium in non-laying female pigeons varied between 8.0 and 8.8 mg. of calcium per 100 cc. of serum, but when daily injections of 1,000 I.U. of estrogen were made, these values increased, ranging from 13.12 to 25.65 mg. of calcium over a period of 10 weeks.

Altmann and Hutt (1938) stated that intramuscular injections of 1,400 R.U. of estrone (theelin) into immature pullets (1.43 kg., 100 days old) over a 14-day period resulted in an increase of blood calcium on the sixteenth day to 16.8 mg., a level 64 per cent higher than in the same birds prior to the test. The per cent increase in all their experiments was calculated from the control reading and the highest reading obtained after injections were made.

The possible influence of ovarian extracts upon the blood calcium level of fowls is reported by Marlow and Koch (1937), who prepared a

non-crystalline purified estrogenic preparation from hog ovaries. These ovarian preparations produced no significant increase in the blood calcium level of fowls.

Altmann and Hutt (1938) injected 60 cc. of fresh egg yolk per bird into the peritoneal cavity of immature pullets (63 days old) over a period of 17 days. Blood calcium of the yolk-injected birds rose steadily and was 34 per cent higher eight days after the first injection and at two weeks, 35 per cent higher than at the start of injections. Twenty days after injections started, the blood calcium levels in these birds had returned to normal. Injections of 100 cc. of yolk intraperitoneally over a period of 19 days into capons produced an increase of 40 per cent on the fifth day, with somewhat lower readings thereafter. The same workers reported that the removal of 15 to 37 cc. of yolk from laying hens caused a significant drop in the level of blood calcium, the decrease varying from 24 to 48 per cent and being directly proportional in different birds to the amount of yolk removed. Controls upon which a mock operation was performed were unaffected, but in hens in which yolk was squeezed from the follicles and left in the body cavity, the serum calcium dropped 23 per cent. These findings are not in keeping with recent work reported by Richert (1939), who stated that a concentrated extract of hen ovaries and of hen ovary follicles does not produce a significant change in the blood calcium level of pullets.

EXPERIMENTAL DATA

Methods of Procedure

All birds used in these experiments were pure Leghorns or cross-breeds containing a large per cent of Leghorn blood. In most instances blood calcium determinations were taken before the experiment started so that experimental and control birds could be paired. In each case the birds were placed for several days under conditions peculiar to the experiment.

The regular Kansas State College poultry ration was fed. Its calcium content was modified in the work dealing with calcium retention. Since Conrad (1939) showed that temperature had an effect upon blood calcium level, an attempt was made to conduct all experiments at a temperature of from 70°F. to 75°F. Temperatures were recorded on a Tycos thermograph. Because a lack of food in the intestines might cause blood calcium levels to fluctuate, it was thought advisable to use all night lights on practically all experiments.

Blood calcium analyses were made by the Koch (1934, p. 153-155) method, and all blood calcium figures represent milligrams of calcium per 100 cc. of blood. The commercial parathyroid extract used was that prepared by Eli Lilly and Company, and contained 100 units per cubic centimeter. The theelin used was that prepared by Parke-Davis and Company, and contained 10,000 International Units (I.U.) or 3,000 Rat Units (R.U.) per cubic centimeter. All control birds used in the

theelin experiments were injected with a vegetable oil corresponding to the amount and method of theelin used.

Experiments with Mammalian Parathyroid Extract

Effect on Blood Calcium. Attention has been called to the conflicting results obtained following injections of parathyroid extract into birds. Since the parathyroid has long been known to be associated with calcium mobilization, a series of experiments was conducted in which dosage, time of bleeding, sex, and physiological condition of the birds were varied.

In Experiment I, four groups of birds, viz., laying hens (1.7 kg.), molting hens (1.7 kg.) which were in their first year of production, immature pullets (1.1 kg., 12 to 14 weeks old), and cockerels (2.1 kg., 16 weeks old) were used. Each group consisted of six control and six experimental birds. Control calcium readings were taken on all birds to facilitate pairing. The experimental birds were injected intramuscularly with .5 cc. of parathormone extract per kilogram of body weight and blood samples were taken for calcium analysis 19 hours after the experimental birds had been injected. This dosage and time of bleeding were selected because Macowan (1932) reported a distinct increase in blood calcium of pullets 12 to 19 hours after a single intramuscular injection of .5 cc. of parathormone.

The results of this phase of work which have been summarized in Table 1 were analyzed statistically. The means were treated by the

Table 1. Effect of parathormone upon blood calcium level of chickens. Summary of data from Experiment I. (Figures given are the mean for each group of six birds.)

Group	Control birds			Experimental birds		
	Number of birds	Mg. Ca/100 cc. of blood		Number of birds	Mg. Ca/100 cc. of blood	
		Beginning of experiment	19 hours later		Before injection	19 hours later
Immature pullets	6	8.44	9.08	6	8.20	8.61
Molting hens	6	9.05	8.35	6	8.82	8.92
Laying hens	6	15.43	16.98	6	17.54	18.30
Cockerels	6	7.64	8.67	6	7.59	8.62

method outlined by Snedecor (1938, p. 56). The "t" scores were determined between controls of the control bleeding and controls of the experimental bleeding; between controls and experimentals of the control period; between controls and experimentals of the test period; and between experimentals of the control bleeding and experimentals of the experimental bleeding. None of the "t" values proved to be significant within the 5 per cent level. These results indicate that mammalian parathyroid extract either exerts no influence on the blood calcium level of the bird, that the dosage was inadequate, or that the time of bleeding following injections did not coincide with the peak of blood calcium elevation.

Experiment II was carried out to determine if large doses of parathyroid extract would raise the blood calcium level in molting hens. Frequent bleedings were made to determine the exact time of increase if there was any rise in blood calcium. Four control and four experimental molting hens (1.7 kg.) were selected. Blood calcium determinations were run, and after waiting one week control readings were again taken on all birds. The experimental birds were then injected subcutaneously with 1.5 cc. of parathormone per kilogram body weight. Blood calcium determinations were run on all birds at intervals of 2, 4, 6, 10, 19, and 24 hours after the experimentals had been injected. This particular dosage and time of bleeding were selected because Knowles, Hart, and Halpin (1935) reported that single subcutaneous injections of 1.5 cc. of parathyroid extract (Lilly) produced a rise in blood calcium; the peak was reached in from $3\frac{1}{2}$ to 8 hours after injection.

Table 2. The effect of parathormone injections on blood calcium of molting hens. Summary of data from Experiment II. (Figures represent mean values for four birds.)

Time of reading	Mg. Ca/100 cc. of blood	
	Control birds	Experimental birds
1 week before experiment was begun	10.40	9.29
Immediately prior to injection	9.28	9.05
2 hours after injection	10.35	10.34
4 " " "	10.39	10.91
6 " " "	10.74	11.12
10 " " "	11.22	11.22
19 " " "	12.30	10.71
24 " " "	12.36	11.38

The data from Experiment II, as recorded in Table 2, show that the calcium level in the experimental birds failed to rise above that of the control birds. The data also show that except for a single instance the level of blood calcium at each subsequent bleeding after injection in both controls and experimentals was slightly higher than that of the preceding bleeding. This emphasizes the need for positive controls, and suggests that hemorrhage or handling tends to increase blood calcium levels. These results would suggest that a large single dose of mammalian parathyroid extract (1.5 cc./kg.) has no apparent effect on blood calcium of molting hens.

Because no increase in blood calcium was secured with injections of parathyroid extract over a short period of time, as is shown in Table 2, it was decided that possibly daily injections of parathyroid extract over a period of several days might be required to demonstrate

an increase in blood calcium. Accordingly, in Experiment III, four control and four experimental molting hens (1.7 kg.) were selected. Control blood calcium determinations were taken on all birds. The experimental birds were then injected subcutaneously with 1.5 cc. of parathyroid extract per kilogram body weight, which was followed by daily injections of .5 cc. of the extract for six days. At the end of this period blood samples were secured for analysis.

Table 3. The effect of continued doses of parathyroid extract on blood calcium of molting hens. Summary of data from Experiment III. (The birds used in this experiment were the same as those used in the calcium metabolism studies.)

Time of reading	Mg. Ca/100 cc. of blood	
	Control birds	Experimental birds
Immediately prior to injection	9.28	9.05
1 week after injection	13.16	11.49

It is worthy of note that in the above experiment the calcium content of the blood in both control and experimental birds increased slightly, and that the controls increased even more than the experimentals. This further emphasizes the value of positive controls in an experiment of this type. These data are interpreted as indicating that large doses of the parathyroid extract administered over a protracted period will not elevate the level of calcium in the blood of sexually mature hens.

Effect on Calcium Metabolism. As seen from Experiments I, II, and III, the writer has been unable to demonstrate an increase in the blood calcium of the birds of either sex at any stage of maturity by single or repeated injections of mammalian parathyroid extract. A hen fasted to remove dietary calcium from the gut can mobilize in a period of 17 hours sufficient calcium to produce a normal egg shell. A shell weighing 5.5 g. would contain approximately 2.2 g. of calcium. If blood volume is assumed to be 9 per cent of the body weight and its specific gravity is 1.06, and the calcium content of the blood is assumed to be 30 mg. of calcium per 100 cc. of blood, the amount of calcium present at one time in the blood of a 2 kg. hen would be approximately 51 mg. Since dietary calcium is not available for shell formation, it is obvious that the amount of calcium in the blood at one time (51 mg.) is approximately one-fourtieth of the amount needed for a single egg shell containing 2.2 g. of calcium. It must be concluded that the hen can, in the absence of dietary calcium, draw upon her body reserves (bones) for calcium, and that this calcium is excreted during shell formation.

In support of this, Common (1936) found that laying hens on a low calcium intake excreted an abnormal amount of phosphoric acid. The only logical explanation for this is that the hen, drawing upon the calcium phosphate of the bones, excretes the calcium via the egg shell and the phosphorus is liberated via the feces. Moreover, Feinberg, Hughes, and Scott (1937) were able to detect a perceptible increase in

inorganic phosphorus in the blood of the hen during active shell formation. Common (1933) found that calcium retention in a bird's body rose steadily over a period of about two weeks before egg laying began; it attained a level of from 70 to 75 per cent of the intake during the laying period; after laying ceased, the per cent of calcium retention decreased. Common (1938) reported that the calcium oxide content of the pullet's body was raised considerably by feeding a ration high in calcium before laying, and that this calcium oxide must be stored in the bones which contain 97.2 to 98.7 per cent of all the calcium oxide in the body. He showed that the pullet may use up to about one-fourth of its body calcium at the onset of laying for purposes of shell formation. Under these conditions the excretion of calcium was greater than the amount ingested, regardless of the level of calcium in the diet. The pullet thus is in a negative calcium balance and is using previously stored calcium to perform the body functions.

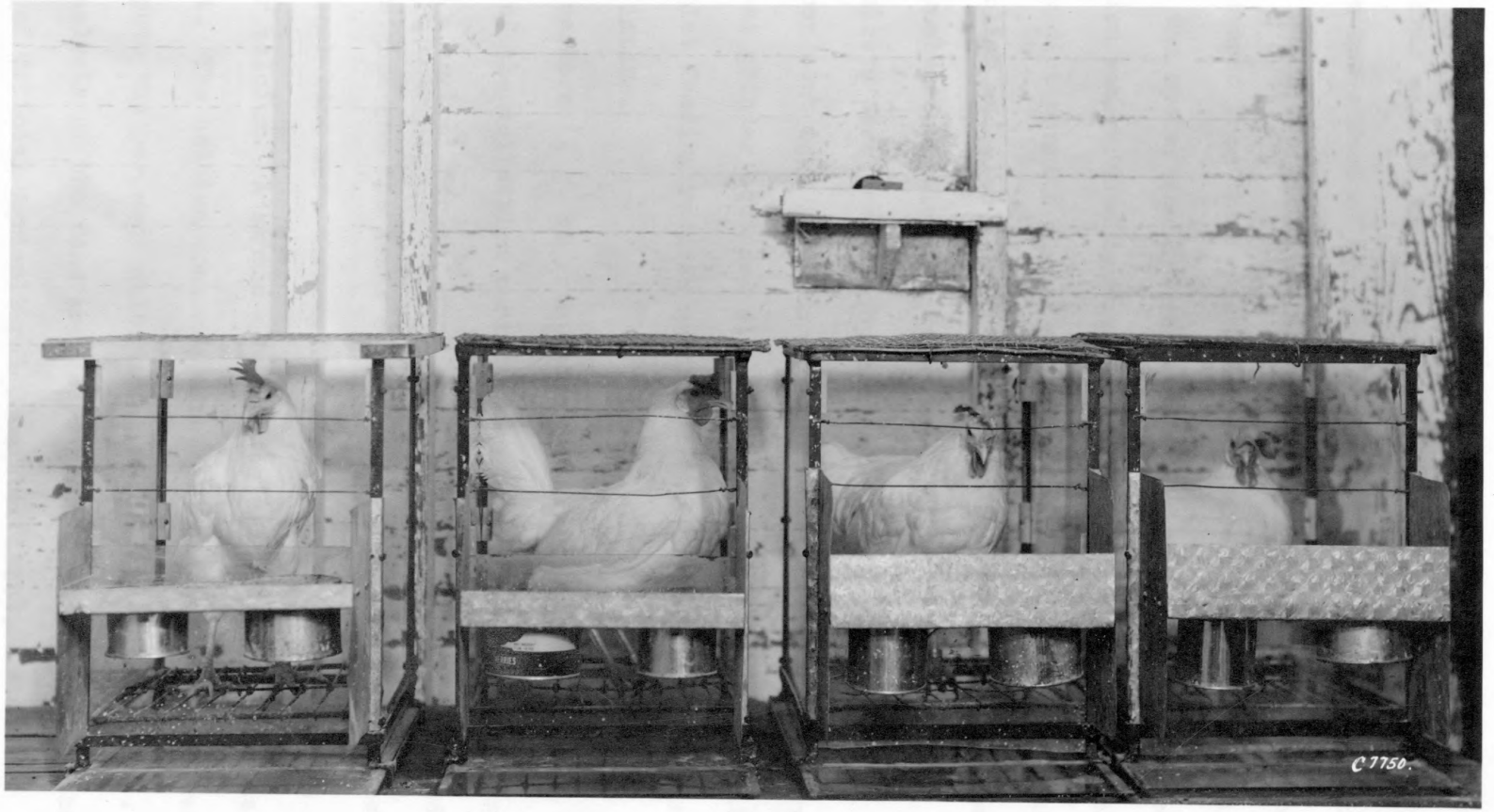
The withdrawal of calcium from the bones is attributed in part to the activity of the parathyroid gland. It remains to be seen if the parathyroid preparation used in the present series of experiments, although failing to elevate the blood calcium level, tended to deplete the body reserves. This function of the parathyroid was tested by means of a calcium balance study (Experiment IV).

Four control and four experimental molting hens (1.7 kg.) were paired and placed in specially constructed metabolism cages which are illustrated in Plate I.

EXPLANATION OF PLATE I

The metabolism cages were constructed to minimize feed wasteage and loss of feces. The frame is made of strap iron which has been welded, and the sides are of glass. The top, which acts as a lid, is made from hail screen. The floor is a grid that is raised high enough from the bottom to prevent the bird from gaining access to its droppings. The feces are collected on a sliding glass bottom which has paraffin ridges around the sides. This prevents feces from running over the sides. All the feeders and waterers were outside the cages and constructed so that neither feed nor water could be wasted. These cages are easily dismantled and prove very satisfactory for retention studies.

Plate I



The birds used in Experiment IV are the same ones used in Experiment III on the effect of continued doses of parathyroid extract, the results of which are given in Table 3. Distilled water and mash pellets prepared from the regular Kansas State College poultry laying mash minus the oyster shell were given the birds. The calcium content of the ration was reduced because Zwarenstein (1934) suggested that the low calcium diet permits a more accurate evaluation of endogenous calcium metabolism and also eliminates to a large extent the unknown factor of unabsorbed calcium in the feces.

The pellets were prepared by mixing 5 pounds of mash with 1 liter of warm 1 per cent agar solution and immediately forcing it through an ordinary meat grinder equipped with a plate containing pellet-size holes. Pellet feeding reduces waste and hence insures a reliable index of calcium intake.

Feces markers (1 g. of iron oxide in capsules) were used to mark the limits of the collection period. The experiment was then run for one week as a control period, during which time the feed ingested was carefully weighed and the feces collected. At the end of the week all feces from each bird were thoroughly dried, ground, and a sample analyzed for calcium. A sample of feed was also analyzed for its calcium content. The calcium consumed and total calcium excreted were used to compute the per cent calcium retained in the bird's body. The method of analysis of the feed and feces was that described by the Association of Official Agricultural Chemists (1935).

At the conclusion of the control period, the experimental birds were given an initial injection of 1.5 cc. of parathyroid extract (subcutaneously) per kilogram of body weight, and .5 cc. per kilogram of body weight daily for the remaining six days.

The amounts of calcium consumed and excreted, and the per cent retention for each bird during the control and experimental period are submitted in Table 4.

Table 4. The effect of parathyroid extract upon calcium metabolism. Summary of data from Experiment IV.

Bird number	First week			Second week		
	Mg. calcium consumed	Mg. calcium excreted	Percent calcium retained	Mg. calcium consumed	Mg. calcium excreted	Percent calcium retained
1 control	8862.0	7724.05	+12.8	6478.5	5468.82	+15.6
1x expt.	8967.0	7741.18	+13.7	6163.5	5221.44	+15.4
2 control	10479.0	9444.55	+ 9.9	11886.0	8314.57	+ 3.0
2x expt.	6930.0	5642.07	+18.6	3591.0	3806.88	- 6.0
3 control	6562.5	5865.68	+10.6	6562.5	5302.92	+19.2
3x expt.	10689.0	9718.80	+ 9.1	7812.0	6228.34	+20.3
4 control	2772.0	1893.36	+31.7	7119.0	4553.01	+36.0
4x expt.	2163.0	1592.56	+26.4	2677.5	1506.96	+43.7

An examination of Table 4 shows that without exception all birds were in a positive calcium balance during the control period and that during the second week or experimental period, with but one exception, all birds were in a positive calcium balance. This would indicate that daily injections of a parathyroid extract exert no influence upon calcium metabolism of molting hens.

Tests with Avian Preparations (Experiment V)

As it was found by Experiments I, II, and III that mammalian parathyroid extracts had no effect upon the blood calcium level of fowls, the question now arises as to whether avian preparations might exert some effect upon the blood calcium level.

A series of tests were conducted in Experiment V in which the effect of avian preparations on the blood calcium level of immature pullets (.8 kg.) was studied. Different endocrine glands were removed from laying hens or cockerels (12 to 14 weeks) and either the whole glands were inserted, or the glands were placed in a small amount of normal saline solution and macerated before being injected. All preparations were injected within a very short time after the fresh materials had been secured from living birds.

The different tests were similar but involved different dosages and time of taking blood samples for calcium analysis after injection. The results have been condensed and are presented in tabular form (Table 5) so that all work on a specific gland can be readily compared.

An examination of Table 5 indicates that injections of avian pituitary, adrenal, and blood preparations had no apparent effect upon the blood calcium level of immature pullets. There was no effect from thyroid preparations except in the case of thyroid injections from three laying hens, which showed a depressing effect on blood calcium in $17\frac{1}{2}$ to $18\frac{1}{2}$ hours after injection. When parathyroid injections

(intramuscular) from three laying hens were made, a slight increase in blood calcium was shown 24 to 27 hours after the injections.

Results as shown in Table 5 indicate that avian preparations have no significant effect upon the blood calcium level of immature pullets. Any response obtained could not be duplicated in subsequent experiments in which similar preparations were used.

Table 5. The effect of avian preparations upon blood calcium of immature pullets. Summary of data from Experiment V.

Number in expt.	Preparation injected	Mg. Ca/100 cc. of blood Hours after injection				
		0	10	15	17½-18½	24-27
1 expt.	Pituitary (3 laying hens)	6.06			6.06	
1 cont.		6.86			6.06	
1 expt.	" (12 cockerels)	7.85			8.97	
1 cont.		7.55			8.78	
1 expt.	" (5 laying hens)	8.63				9.25
1 cont.		8.63				10.05
1 expt.	Adrenal (3 laying hens)	7.68			8.48	
1 cont.		5.66			6.06	
1 expt.	" (12 cockerels)	6.32			8.16	
1 cont.		6.53			7.55	
1 expt.	" (5 laying hens)	9.04				9.04
1 cont.		9.04				8.84
1 expt.	Thyroid (3 laying hens)	8.88			5.63	
1 cont.		6.46			6.86	
4 expt.	" (3 cockerels)	7.90			9.07	
4 cont.		7.21			8.67	
1 expt.	" (5 laying hens)	9.85				10.05
1 cont.		8.63				10.05
1 expt.	Parathyroid[2] (3 laying hens)	6.66		6.48		6.66
1 cont.		6.66		6.66		8.48
2 expt.	" [2] (3 laying hens)	6.66	6.75			6.15
2 cont.		6.86	6.66			6.36
1 expt.	" [3] (3 laying hens)	8.28			9.49	11.11
1 cont.		7.28			7.88	8.28
1 expt.	" (12 cockerels)	8.78			10.20	
1 cont.		8.16			9.58	
1 expt.	" (5 laying hens)	9.25				9.45
1 cont.		8.63				9.04
1 expt.	50 cc. plasma[4] (laying hen)	8.48			7.68	
1 cont.		9.30			7.68	
1 expt.	Plasma from 100 cc. blood (laying hen)	6.26	Died			
1 cont.		8.88		7.28		
1 expt.	Red blood cells from 100 cc. blood (laying hen)	6.68		7.28		

[1] Intramuscular injections.
[2] Whole glands inserted.
[3] Intraperitoneal injections.
All other glands were macerated in a small amount of normal saline solution and injected subcutaneously.

Experiments with Estrogenic Preparations

The results obtained on mammalian parathyroid extract seem to show conclusively that this preparation is not responsible for the increase in blood calcium associated with age at sexual maturity. It seemed advisable to investigate the possible influence of estrogens since the response following injections of these substances into birds is variable.

In Experiment VI, five control and five experimental molting hens (2 kg.) were selected to ascertain the effect of theelin administered over a protracted period upon the blood calcium level. Control calcium values were obtained in advance of the injections.

All experimental birds were then daily injected intramuscularly with 85 R.U. of theelin per kilogram of body weight for nine days, and 255 R.U. daily for the remaining six days. Seven blood samples for analysis were taken at intervals during the 15-day period. The results of this experiment are summarized in Table 6.

Table 6. Effect of injections of theelin on blood calcium of molting hens. Summary of data from Experiment VI. (Figures represent mean values for five birds.)

Days after injection	Mg. Ca/100 cc. of blood	
	Control birds	Experimental birds
0	13.63	14.48
2	18.34	17.77
4	22.27	22.21
6	25.79	22.10
9	22.55	16.00
13	23.13	20.80
16	22.07	18.01

The data show that there was a rise in blood calcium of all birds, but that the increase of the controls exceeded that of the experimentals. For these reasons it was decided to conduct an experiment similar to the last one but to use immature pullets and to inject larger doses of theelin.

For Experiment VII, ten control and ten experimental immature pullets (14 weeks old) averaging .8 kg. body weight were selected and paired. Two control bleedings for blood calcium analysis were taken, one four days before injections started, and the second just before the experimentals were injected. Experimental birds were injected subcutaneously every second day with 250 R.U. (125 R.U. per day) of theelin per kilogram of body weight for 14 days. This was nearly twice the amount injected by Altmann and Hutt (1938), who reported a rise in blood calcium following injections over a 14-day period of 1,400 R.U. of theelin into immature pullets. Blood calcium samples were taken every third day following initial injections.

Because no apparent rise in blood calcium was found at the end of the 15-day period, it was decided to inject each experimental with 1,000 R.U. of theelin per kilogram of body weight daily for four days. This was followed with but one exception, in which case the bird was injected with 1,000 R.U. per kilogram of body weight for the 4-day period plus an additional 6,000 R.U. daily for the last two days. At the end of that period, this bird, two other experimental birds, and three controls were killed for histological studies. The remaining 14 birds in the experiment were bled two and six days after final injections were made. Table 7 gives the pertinent facts concerning the results of Experiment VII on immature pullets.

Table 7. Effect of continued massive doses of theelin on the blood calcium of immature pullets. Summary of data from Experiment VII.

	Controls		Experimentals		
Days after injection	Number in expt.	Mg. calcium per 100 cc. blood	Number in expt.	Total R.U. theelin/kg. body weight	Mg. calcium per 100 cc. blood
-4	10	9.94	10	0	9.97
0	10	9.63	10	0	9.53
3	10	7.91	10	250	8.06
6	10	8.08	10	625	8.32
9	10	9.50	10	1,000	10.25
12	10	8.47	10	1,375	9.03
15	10	8.55	10	1,750	9.63
19	9	7.88	9	5,750	14.98
21	6	8.60	6	5,750	10.98
25	6	7.64	6	5,750	9.78
19	1	7.53	1	17,750	15.25

An analysis of the results shown in Table 7 indicates that injections totaling 1,750 R.U. of theelin over a 15-day period (125 R.U. per day) have no effect upon the blood calcium level of immature pullets. However, when massive doses (1,000 R.U. daily for four days) of theelin were injected into immature pullets that had previously received 1,750 R.U. of theelin over a 15-day period, there was a distinct rise in the blood calcium level, but in no case did this rise approach that of a laying hen. It is interesting to note that when larger dosages (6,000 R.U. daily for two days) were given there was no further increase in the blood calcium level. Blood calcium values had approached their former level six days after the final injection.

It is worthy of note that the experimental birds described in Table 7 showed marked changes in the comb and spread of pubic bones, the combs of the experimentals becoming enlarged and showing improved circulation. The pubic bones tended to spread, and the pullets in general took on characteristics similar to a hen approaching production. Three control and three experimental birds were killed after they had been injected with 5,750 R.U. of theelin over a 19-day period. That the dosage of theelin injected was sufficient to cause a marked degree of hypertrophy in the oviduct is indicated by the photomicrographs of Plate II.

EXPLANATION OF PLATE II

Fig. 1. Cross section of oviduct of control bird. 40X. Photomicrograph.

Fig. 2. Cross section of oviduct of experimental bird injected with 5,750 R.U. of theelin over a 19-day period. 40X. Photomicrograph.

Plate II

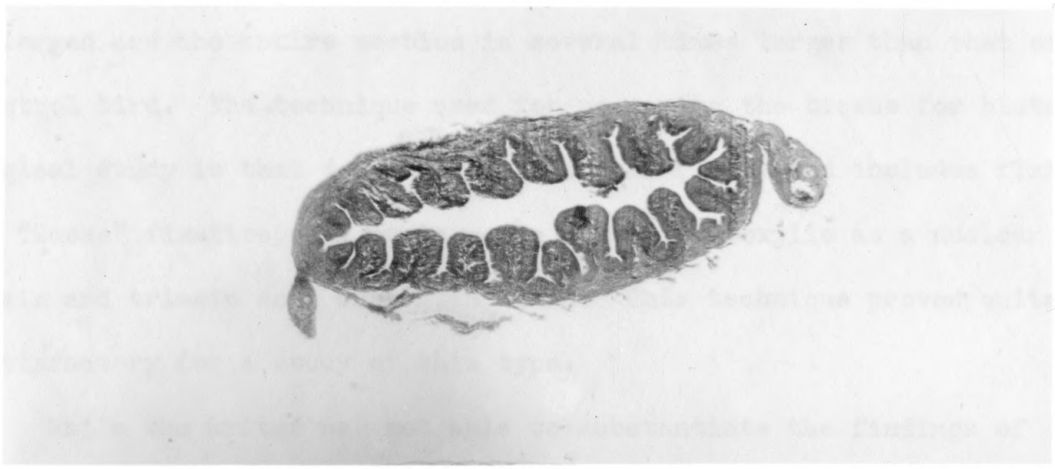


Fig. 1

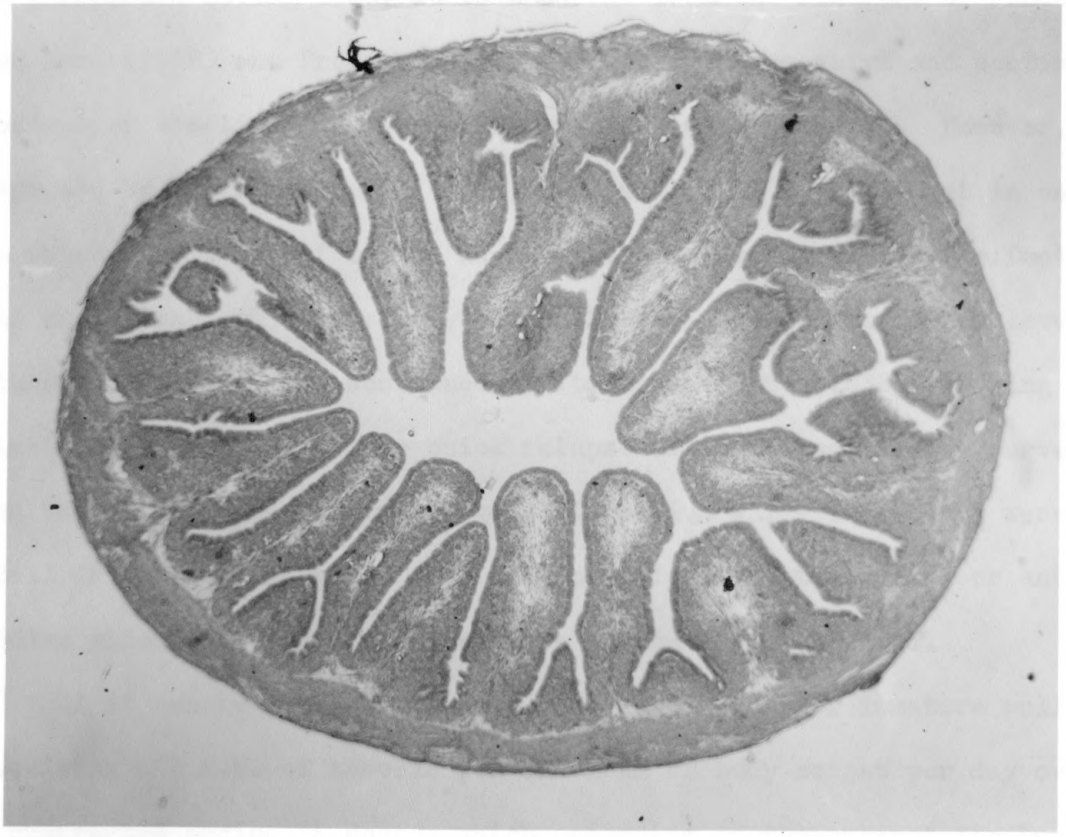


Fig. 2

The folds of the magnum containing the tubular glands are much enlarged and the entire section is several times larger than that of a control bird. The technique used for preparing the tissue for histological study is that described by Galigher (1934) and includes fixing in "Susas" fixative, and staining in alum haematoxylin as a nuclear stain and triosin as a chromatin stain. This technique proved quite satisfactory for a study of this type.

While the writer was not able to substantiate the findings of Altmann and Hutt (1938) by giving nearly twice the dosage of theelin they reported having used, it is apparent from the findings of Zondek and Marx (1939) and from the present results that massive and continued dosages of theelin give a response in blood calcium level. However, as reported by Levin and Smith (1938) in mammals, it appears that in cases in which huge doses of estrin had a statistically significant effect, the change was only transitory since calcium returned to former levels although treatment was continued. Altmann and Hutt (1938), working with birds, felt that the rather quick relapse of the blood calcium curve and the resumption of normal levels of calcium while injections were still in progress resulted from the formation of antihormones or antibodies which counteracted the effects of the agent injected.

If it can be shown that the blood and/or urine of immature pullets receiving 125 R.U. of theelin per kilogram of body weight per day over a protracted period, a dosage which according to the present findings is not sufficient to elevate blood calcium, contains an estrogenic

substance in quantities as great or greater than that contained in the blood or urine of a laying hen, it would show conclusively that theelin is not the agent normally responsible for the increase in blood calcium. To make these tests Experiment VIII was conducted.

Five immature pullets averaging .9 kg. each were injected daily with 125 R.U. of theelin per kilogram body weight for a period of 20 days. Blood samples for calcium analysis were taken at 0-, 5-, 10-, and 15-day intervals following date of initial injection. Mean calcium values of 8.47, 8.63, 8.38, and 8.52 per 100 cc. of blood were obtained for the intervals specified.

The estrogenic activity of the blood was assayed after 5 and 15 daily injections. The blood and urine from laying hens was also assayed for estrogens. The assays of blood and urine from the pullets and laying hens used in Experiment VIII are presented in Table 8.

An examination of Table 8 indicates that there is no estrogenic effect from blood or urine of laying hens and that it is very questionable if there is any response following the injection of blood or urine from pullets which themselves had been injected with theelin. All assay work on blood and urine was done in cooperation with Dr. H. W. Marlow, Department of Chemistry, Kansas State College of Agriculture and Applied Science.

Table 8. The assay of chicken blood and urine for estrogenic substances. Summary of data from Experiment VIII.

Preparation injected	Amount injected (cc.)	Hours after injection into rat: 0	41	44	45	47	48	53	54	55	60	65	69	70	72	90
Blood serum from laying hen	1	5[5]	5			5						5				5
" " " " "	2	5	5			5						5				5
" " " " "	3 + 2 (41 hrs.)	5	5			5						5				5
" " " " "	4 + 4 (4 hrs.)	5	-			5		5					5			-
" " " " "	4 + 1 (10½ hrs.)	5	-			5		5					5			-
Urine from laying hen	3	5							5			5				
" " " "	4	5							5			5				
" " " "	5	5							5			5				
" " " "	9	5							5			5				
" " " "	17	5		5			5		5		5	5				
Blood serum from pullet[7]	1	5				5				5					5	
" " " "	2	5				5				5					5	
" " " "	3	5				5				5					5	
" " " "	4	5				5				5					5	
" " " "	3 + 4 (4 hrs.)	5				5				5					5	
Blood serum from pullet[8]	1	5			5		5		5		5			5		
" " " "	2	5			5		5		5		5?			5		
" " " "	4	5			5		5		5?		5			5		
" " " "	6½	5			5		5?[6]		5?		5			5		
Urine from pullet[8]	3	5							5?			5?				
" " "	4	5							5			5				
" " "	5	5							5?			5?				
Urine from pullet[9]	4	5		5			5		5		5					
" " "	6	5		5			5		5		5					
" " "	8	5		5			5		5		5					
" " "	10	5		5			5		5		5					

[5] Indicates normal vaginal smear.
[6] Indicates slight question as to smear being normal.
[7] Sample after 5 daily injections of 125 R.U. of theelin per kilogram of body weight.
[8] Sample after 15 daily injections of 125 R.U. of theelin per kilogram of body weight.
[9] Sample after 20 daily injections of 125 R.U. of theelin per kilogram of body weight.

If estrogens are present in the blood or urine of laying hens, the amounts are so small that they cannot be assayed without concentration and fractionation. The failure of theelin to appear in the urine of pullets receiving massive doses of it would suggest that the fowl is capable of modifying the material so as to inactivate it.

DISCUSSION

Attention has been focused upon the contradictory claims of causes for an increase in the blood calcium level of the fowl at the time of ovarian activity. During the present series of experiments, special attention was given to the large number of factors, environmental and otherwise, that might influence the level of calcium in the blood of birds.

Macowan (1932) reported a distinct rise in the blood calcium of immature pullets from 15 to 19 hours after intramuscular injections of .5 cc. of parathyroid extract, but failed to find a similar response with molting hens, although the dosage was twice as great. This latter result is in keeping with the present findings.

Knowles, Hart, and Halpin (1935) reported an increase in blood calcium of immature pullets and molting hens when 1 to 3 cc. of parathyroid extract were given; the peak of the rise came 2 to 10 hours after injections. They failed to produce a significant response in capons or cocks which had received similar doses.

Altmann (1938) injected 2 to 4 cc. of parathyroid extract intra-

muscularly over a 2-day period into immature pullets and produced a 47.17 per cent increase in blood calcium. The positive controls rose 20.46 per cent.

The results of the present experiments fail to show a significant increase in the blood calcium of molting hens, laying hens, immature pullets, or cockerels which had been injected intramuscularly with .5 cc. of parathyroid extract per kilogram body weight. Large doses of parathyroid extract (1.5 cc. per kilogram of body weight) failed to elevate the blood calcium of molting hens in 2, 4, 6, 10, 19, or 24 hours after injection. Subcutaneous injections of parathyroid extract (1.5 cc. for first day and .5 cc. for six days) failed to elevate the blood calcium level, or to exert any influence upon calcium retention.

Injections of avian parathyroid, pituitary, adrenal, thyroid, and blood preparations into immature pullets failed to produce a significant increase in their blood calcium level but did not depress the level of blood calcium.

The present results on mammalian parathyroid preparations and certain crude avian endocrine preparations indicate that these preparations are not responsible for the increase in blood calcium associated with age at sexual maturity.

The results obtained with the estrogenic substance theelin did not agree fully with the findings of other workers. This work showed that intramuscular injections of 2,100 R.U. of theelin per kilogram of body weight over a 15-day period failed to increase significantly the blood

calcium level of molting hens. Altmann and Hutt (1938) reported that injections of 1,400 R.U. of estrone (theelin) into immature pullets (1.43 kg., 100 days old) over a 14-day period resulted in an increase in blood calcium on the sixteenth day to 16.8 mg., a level 64 per cent higher than in the same birds prior to the test. The data from the present experiments indicate that 1,750 R.U. of theelin per kilogram of body weight injected into immature pullets (.8 kg.) over a 15-day period had no effect upon the blood calcium level, but when larger doses (5,750 R.U. per kilogram) were injected over a 19-day period, there was an increase in the blood calcium. The control birds averaged 7.88 mg. of calcium and the experimentals 14.98 mg. of calcium per 100 cc. of blood at the end of this 19-day period. When these massive dosages of theelin were discontinued, the blood calcium in the experimentals returned to normal levels within six days.

The present results would indicate that estrogens are not normally responsible for the elevation of blood calcium in the fowl during egg production and that the assay experiment set up to test this event proved non-enlightening.

SUMMARY

1. Studies of possible causes for blood calcium elevations in fowls were carried out with various endocrine and other preparations on approximately 150 chickens.

2. Varying doses of a mammalian parathyroid preparation failed to

elevate the blood calcium level of either sex at any age, nor was there any indication as a result of a calcium balance study that the bird depleted its body of calcium reserves.

3. Crude avian parathyroid preparations, as well as similar preparations from other avian endocrines such as the pituitary, thyroid, and adrenals, appeared to exert no influence on the blood calcium level of immature pullets.

4. A slight increase was observed in the blood calcium level of immature pullets following injections of massive doses of theelin (5,750 R.U.) over a period of 19 days.

5. A rat assay of blood and/or urine from laying hens, as well as that from immature pullets which had been injected with 125 R.U. of theelin per kilogram of body weight per day, indicated that these body fluids (blood and urine) do not contain detectable quantities of estrogen.

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