ADRENAL CONTROL OF BLOOD CALCIUM IN THE HEN

by

LOWELL WILLIAM TAYLOR

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INTRODUCTION

For many years physiologists have recognized that the calcium metabolism of the laying hen is unusually complex. Investigators pointed out that the calcium level varies from eight to 25 mg percent and that the level even fluctuates hourly. These workers have been of the opinion that this peculiar phenomenon is under endocrine control, but in spite of many attempts they have been unable to alter the blood calcium level of the domestic hen with any recognized hormone unless administered in massive dosages.

At least a partial explanation of this peculiar phenomenon has rapidly progressed in very recent years. A group of workers at Kansas State College experiment Station (unpublished) while working on the effect of temperature in the laying hen, observed that saline extracts of pullet and mammal adrenals lowered the calcium level in the laying hen much the same as a rise in temperature.

Other investigations suggest that some factor in the adrenals inhibits parathyroid activity in the mammal, and that high temperatures in some way inhibit the activity of parathyroid extracts, resulting in a lower calcium level.

This factor would not only prove valuable in explaining blood calcium control in the laying hen, but it might also be instrumental in treating hyperparathyroidism in the mammal, which has so far been untreated with the exception of partially removing the parathyroid, or giving X-ray treatment.

From these considerations it has seemed desirable to study the physical and chemical properties of the adrenal hormone so that preparations can be made for a further study of its physiological function.

REVIEW OF LITERATURE

First investigations on extensive studies of the effects of various endocrine factors on blood calcium in the laying bird were inspired by the work of Riddle and Reinhart (1926), and Hughes, Titus and Smits (1927). These men demonstrated that the calcium levels in the laying hen are higher than in the non-laying hen and that this blood calcium level fluctuates hourly.

Some workers have shown that estrogens raise blood calcium in the fowl, others have found them to have no effect, and in one case a lower blood calcium was found after administration. The discrepancies can probably be attributed to differences in dosage, temperature changes, diet and the method used in determining blood calcium.

Elevation of the calcium level following estrogen administration has been reported by several groups of workers. Using pigeons, doves and fowl, Riddle and Dotti (1934, 1938) found that estrone was more effective than estriol or estradiol in raising the blood calcium level. These same conclusions were reached by Zondek and Marx (1939) who in addition concluded that the synthetic compound diethylstilbesterol also produced

calciema in chicks of both sexes. Experimenting on egg yolk, Altmann and Hutt (1938) reported that 50 to 100 ml injected over a period of eight to 19 days into immature fowls and capons produced a significant rise in serum calcium; they also reported that 500 R.U. of progynon B administered over a period of nine days gave a maximum increase of 25 percent, while 14,000 R.U. of estrone given over a period of 14 days increased blood calcium 64 percent by the 16th day. Landauer (1940) using adult cocks and drakes; Landauer, Pfeiffer, Gardner and Man (1939) using cocks; Pfeiffer and Gardner (1938) using pigeons; and Pfeiffer, Kirschbaum and Gardner (1940) using the English sparrow, all observed calcemia following estrogen administration.

On the other hand many groups of investigators were unable to produce a significant calcium change after estrogen administration. However these groups used smaller dosages. It appears that these dosages were still larger than the amount secreted by the normal laying hen, if the results of Marlow and Richert (1940) are accepted. These show that not more than five R.U. of estrogens are obtained per hen ovary by their method of extraction.

Using pullets and cocks, Marlow and Kock (1937) found that non-crystalline, purified estrogenic products, and preparations from hog ovaries and bull testes, failed to produce a significant and constant effect on blood calcium level. Marlow and Richert (1940) obtained identical results following injections of avian ovarian extracts. Avery, Scott and Conrad (1940) observed no significant change in serum calcium 19 hours

after injecting 2295 R.U. of theelin per kilogram body weight in molting hens. They also found that 1750 R.U. of theelin per kilogram of body weight, given over a 15 day period, did not significantly raise blood calcium in pullets, but that 5750 R.U. over a period of 19 days did produce calcemia.

Work has been done using parathyroid hormone, which plays an important role in calcium metabolism in the mammal. Calcemia was produced in some cases and in others the calcium level was not significantly altered. In 1932, Macowan demonstrated that parathyroid extract injections elevated blood calcium from two to 6.5 mg percent in pullets, but not in moulting hens or cockerels. Knowles, Hart and Halpin (1934), and Deobald, Lease, Hart and Halpin (1936) observed calcemia in immature pullets and non-laying hens, but not in cocks and capons when one to three milliliters of Lilly parathyroid preparation were given. Altmann (1938) reported an increased blood calcium level of 47.17 percent following injections of 2.4 ml of Squibb and Sons parathyroid extract, but Collip (1931) was unable to demonstrate any effect on blood calcium level in the non-laying hen after injecting parathyroid extract. Injecting as much as 1.5 ml of Lilly preparation per kilogram of body weight, Avery, Scott and Conrad (1940) were unable to alter calcium level in moulting hens, laying hens, immature pullets or cockerels. Campbell and Turner (1942) were unable to raise blood calcium level in either chicks two to three days old, or one month old. Injections were 0.25 ml of Lilly extract per bird.

If mammalian parathyroid extracts do not affect blood calcium in the fowl these negative results may be due to species specificity. It is hard to conceive of the presence of a parathyroid in an animal which does not affect the blood calcium level. So far there are no reports that avian parathyroid extracts have been injected into the fowl.

Lowered blood calcium in the laying fowl and some mammals has been found to be due to high temperatures; experiments also show that high temperatures inhibit the effect of injections of parathyroid extracts. Conrad (1939) was successful in showing that an increase in temperature from 70 to 90 degrees decreased blood calcium approximately 30 percent in laying hens.

It has been found that during high fevers the blood calcium level is considerably lower and parathyroid injections are relatively ineffective in bringing the calcium level back to normal. Linder (1935) reported that serum calcium was low in typhoid and response was small to injections of parathyroid extracts, but larger response was obtained on recovery. He suggested that the causes were the inactivation of the parathyroid hormone, and changes in the activity of the parathyroid, thyroid and anterior pituitary. Friedrich (1924) found the blood calcium level in healthy children to be 12 mg percent, but only 10.74 mg percent during chicken pox, 9.35 mg percent during measles, and 10.25 mg percent during scarlet fever. He attributed the drop to high fever. Combes (1940) found that dogs injected with ten units of parathyroid extract per day over an extensive period of time, experienced a significant raise in blood calcium, but on warm days it had little or no

effect.

Several investigators have reported changes in blood calcium following injections of adrenal extracts and following adrenalectomy.

Mirvish and Boxman (1929) lowered blood calcium in rabbits by injecting 30 to 80 g of a fat soluble adrenal cortex extract. They found that the parathyroid must be present for response, and believed that the cortex factor antagonizes the parathyroid. Perhaps the activity obtained from such a massive dosage is only due to impurities. Taylor and Craven (1927) reported that calcium was lowered by adrenal extracts (extraction method not given) and also found that the presence of the parathyroid was necessary for this effect. A group of investigators at Kansas State College Experiment Station (unpublished) lowered blood calcium 30 to 40 percent in laying hens, with saline extracts of the adrenals of hogs, sheep, cattle and fowl.

Taylor and Craven (1927) observed that adrenalectomized cats and dogs experienced elevated blood calcium three to five hours following operation, and Rogoff and Stewart (1928) confirmed this work using adrenalectomized dogs. Donati (1938) and Kisch (1924) observed a high blood calcium in adrenalectomized rabbits, after an initial drop, and Helve (1940) stated that serum calcium is somewhat higher in adrenalectomized rats.

A relationship between the adrenals and the parathyroids is strongly indicated by the work of Schour and Rogoff (1936). They found that the removal of the adrenals or injections of parathyroid extracts identically disturbed the calcification

of dentine in the incissors of the rat.

It appears, as several authors have suggested, that the adrenals secrete a hormone which inhibits parathyroid activity, and it appears that a raise in temperature augments the secretion of this hormone, bringing about a low calcium level in mammal and fowl in warm weather and during fever. The action of this hormone should favor calcium deposition in bone at the expense of blood calcium, there fore producing a thin egg shell in the summer.

EXPERIMENTS

The present study is an extension of previous unpublished work of the poultry physiology group of the Kansas Agricultural Experiment Station, with the aim of describing more completely the blood calcium lowering principle of the fowl adrenal.

The properties studied were: solubility in benzene; solubility in absolute alcohol; effect of boiling for 15 minutes at pH 5.5, 7.0, 8.0 and 9.2; urea denaturation; alcohol denaturation; and effect of irradiating with ultra-voilet light and ultrafiltration.

Assays were made using white leghorn laying hens, fed on a diet ample in calcium and vitamin D, and maintained in batteries at constant temperature, which gradually increased with the season from 65° to 90° F.

Extracts of fowl adrenals[1] were made, treated in various ways and injected intramuscularly into laying birds. A similar number of birds was also injected intramuscularly with an equivalent amount of the untreated extract and used as positive controls. Blood samples were taken from the wing preceding injections, and 24 hours later samples were again taken. Calcium levels were then determined by the Wang (1935) method. Whether this factor had been altered by treatment was determined by the difference in calcium lowering in the birds receiving control and the birds receiving the treated sample.

Preparation of Extracts of Adrenal Glands

Extract 1. The control extract was made by mixing 10 g of well ground glands with 100 ml of normal saline for 30 minutes and centrifuging. One milliliter of the supernatant liquid was injected into each of six birds with the result of lowering the calcium an average of 18 percent.

Extract 2. A benzene extraction was made by treating four grams of ground glands with dry, thiophene free benzene, distilling off the benzene in vacuo, adding more benzene and distilling in vacuo again. This process was repeated until the glands were free of water (the distillate no longer had a milky appearance). The dry residue was then mixed thoroughly for two hours with 20 ml of thiophene free benzene and filtered; the residue was again extracted with thiophene free benzene as above and the

[1] The fowl adrenals were taken from freshly killed pullets and immediately put into a sharp freezer. These glands were furnished through the courtesy of the Fairmont Creamery Co., Omaha, Nebraska.

filtrates were combined. To this combined filtrate was added an equal volume of normal saline; the mixture was then distilled in vacuo to a volume such that 10 ml of extract was equivalent to one gram of glands; this was done to obtain a water solution of this benzene extract. As this water solution had some insoluble fatty material in it which floated to the top, the sample was well mixed before each injection. One milliliter injected into each of six birds lowered the calcium level by an average of three percent.

Extract 3. This extract was prepared just as was Extract 1. One milliliter injected into each of six birds lowered the calcium level by an average of 21 percent.

Extract 4. To two grams of ground glands were added 20 ml of thiophene free benzene; the mixture was shaken for two hours, filtered and the filtrate then added to an equal volume of normal saline. This mixture was distilled in vacuo to such a volume that 10 ml was equivalent to one gram of glands, to free it from benzene. This aqueous mixture contained suspended fatty material and was mixed well before each injection. The injection of one milliliter into each of six birds raised the calcium an average of one percent.

Extract 5. This extract was prepared as was Extract 1. One milliliter was injected into each of six birds with the result of lowering the calcium level an average of 24 percent.

Extract 6. Two grams of ground glands were mixed for two hours with 20 ml of redistilled absolute ethyl alcohol and centrifuged. One milliliter of the supernatant liquid injected into

each of six experimental birds lowered the calcium level by an average of 14 percent.

Extract 7. The extract was prepared as was Extract 1. The calcium level was lowered an average of 16 percent after the injections of one milliliter per bird.

Extract 8. The extraction was made by mixing two grams of ground glands with 10 ml of absolute ethyl alcohol for two hours, centrifuging, re-extracting the residue with 10 ml of absolute ethyl alcohol and again centrifuging. The filtrates were then combined, added to an equal volume of normal saline and distilled in vacuo to a volume such that 10 ml were equivalent to one gram of glands; this was done to partially remove the alcohol from the extract. One milliliter injected into each of six birds lowered the calcium level by an average of four percent.

Extract 9. This extract was prepared just as was Extract 1. The injection of 0.5 ml into each of six birds caused a 16 percent lowering of the calcium level.

Extract 10. A buffer at pH 7.0 was prepared by mixing a 0.1 molar solution of citric acid and 0.2 molar solution of disodium phosphate in proper proportions. Five milliliters of this buffered solution were added to 5.0 ml of Extract 1, the solution was boiled for 15 minutes at 100° C. and filtered through a Whatman, grade 40 filter paper. One milliliter was injected into each of six birds with the result of lowering the calcium an average of 14 percent.

Extract 11. This extract was prepared as was Extract 10. The injection of one milliliter into each of six birds lowered

the calcium level an average of 14 percent.

Extract 12. This extract was identical with Extract 11, except that the pH of the buffer was 5.5. One milliliter injected into each of six birds increased the calcium level by an average of five percent.

Extract 13. This sample was prepared just as was Extract 10. Three and one half milliliters were injected into each of six birds, with the result of lowering the calcium an average of 19 percent.

Extract 14. This extract was prepared just as was Extract 12. The injection of 3.5 ml into each of six birds lowered the calcium level by an average of 10 percent.

Extract 15. A buffer at pH 8.0 was prepared by mixing in proper proportions, a 0.1 molar solution of citric acid and a 0.2 molar solution of disodium phosphate. To 5.0 ml of this buffer were added 5.0 ml of a saline extract prepared just as was Extract 1. This solution was boiled for 15 minutes then filtered through a Whatman, grade 40 filter paper. One milliter of the filtrate was injected into each of six birds with the result of lowering the calcium an average of 10 percent.

Extract 16. A buffer of pH 9.2 was prepared by mixing proper portions of a 0.1 molar ammoniom hydroxide solution with a 0.2 molar ammonium chloride solution. Five milliliters of the buffer were then mixed with 5.0 ml of a saline extract, prepared as was Extract 1.; this solution was then boiled for 15 minutes at 100° C. and filtered through a Whatman, grade 40

filter paper. One milliliter injected into each of six birds raised the calcium level by an average of nine percent.

Extract 17. Work was done on alcohol denaturation, but no controls were run on the same day; however, several days preceding and succeeding controls are very nearly the same, so the control just preceding this work was chosen. This control was prepared as was Extract 1. One milliliter was injected into each of six birds with the result of lowering the calcium by an average of 16 percent.

Extract 18. Two grams of ground glands were mixed for two hours with absolute ethyl alcohol. To dry the glands, the alcohol was distilled off at room temperature; they were then extracted for 45 minutes with 10 ml of a normal saline solution and filtered through a Whatman, grade 40 filter paper. The extraction was repeated on the residue and the filtrates were combined. One milliliter injected into each of six birds lowered the calcium level by an average of five percent.

Extract 19. Eight grams of ground glands were mixed with 40 ml of a normal saline solution for 30 minutes, centrifuged, the supernatant liquid boiled for 10 minutes, this was filtered through a Whatman, grade 40 filter paper and then through a collodion filter under a pressure at 80 pounds per square inch. This extract stood at room temperature for five hours. The injection of one milliliter into each of six birds lowered the calcium an average of 21 percent.

Extract 20. A portion of Extract 19 was exposed to ultraviolet light for five hours at room temperature. This solution

was then heated to 100° C. for two minutes immediately following irradiation, to break the hydrogen bonds, which may still have been holding the main polypeptid chain in its original position. One milliliter injected into each of six birds lowered the calcium level by an average of one percent.

Extract 21. Eight grams of ground glands were mixed with 40 ml of a normal saline solution for 30 minutes, the mixture was centrifuged, the supernatant liquid was boiled 10 minutes and then filtered through a collodion filter under pressure. This control was allowed to stand four hours at room temperature. Injections of one milliliter into each of six birds resulted in lowering the calcium an average of 21 percent.

Extract 22. Eight grams of ground glands were mixed with 40 ml of a normal saline solution for 30 minutes, the mixture was centrifuged, the supernatant liquid was boiled for 10 minutes, filtered through a Whatman, grade 40 filter paper, then filtered through a collodion filter under pressure. To 6.0 ml of this solution were added 3.6 g of urea, and the solution was permitted to stand at room temperature for four hours. The volume was then brought to 9.0 ml. One and five tenths milliliters were injected into each bird with the result of lowering the calcium an average of seven percent.

Extract 23. To four grams of well ground glands were added 15 ml of a normal saline solution, this was mixed thoroughly for 30 minutes, boiled for 10 minutes at 100° C. and filtered through a Whatman, grade 40 filter paper. This solution stood

at five degrees centigrade for five hours. One half milliliter of this extract and also a solution of urea, equivalent to 0.3 g of urea per bird, were injected into each of 12 birds with the result of lowering the calcium an average of 15 percent.

Extract 24. To four grams of well ground glands were added 15 ml of a normal saline solution, this was mixed thoroughly for 30 minutes, boiled for 10 minutes at 100 degrees and filtered through a Whatman, grade 40 filter paper. To 3.0 ml of this solution were added 1.8 g of urea, and the solution was then diluted to 6.0 ml and 1.0 ml was injected into each bird. The calcium was lowered by an average of 10 percent.

Extract 25. Each of six birds was injected with 1.0 ml of a urea solution containing 0.6 g of urea per ml. The calcium was lowered by an average of 16 percent.

Extract 26. Six grams of ground glands were mixed with 40 ml of a normal saline solution for 30 minutes, the mixture was centrifuged, the supernatant liquid was boiled for 10 minutes and then filtered through a Whatman, grade 40 filter paper. One milliliter injected into each of 11 experimental birds lowered the calcium level by an average of 15 percent.

Extract 27. A portion of Extract 26 was filtered through a collodion membrane, through which hemoglobin would not pass, under a pressure of 80 pounds per square inch. One milliliter of this water clear filtrate was injected into each of 12 experimental birds with the result of lowering the calcium an average of 12 percent.

DISCUSSION OF RESULTS

The results of these experiments are expressed in terms of percent change in the calcium level 24 hours after injection. The results were then treated statistically by a method given by Snedecor (1940, p. 58). Calcium change, difference of means, standard error of means and the "t" values are recorded in Table 1. The level of significance of the effect of the treatment is also indicated in Table 1 by the approximate odds against the differences being due to chance, as determined from the "t" values and the number of birds involved.

It will be observed that as this work proceeded, response became less in identical controls; this can probably be attributed to the glands losing activity on standing, to temperature increasing with the season, and to the birds building up an immunity to the hormone. Maximum calcium lowering was obtained when fresh birds and glands were used and while temperature could be maintained at approximately 65° F. It appeared that the glands maintained their activity for at least five months if kept in a sharp freezer.

This work demonstrates that this calcium lowering hormone is insoluble in benzene and alcohol, and the effect of the saline control extracts is evidence that it is soluble in normal saline solution.

Many hormones found in the adrenals, concerned with mineral metabolism, are sterols, but the above solubility phenomena would exclude any possibility of this hormone being a steroid

Table 1. Results of experiments to test the effects of treatments on the adrenal blood calcium lowering hormone of the adrenal gland.

Extract	Date	Treatment	No. birds	Calcium change %	Difference of means %	Standard error of means %	"t" value	Levels of significance %
1	1-15-43	Control	11	-20				
3	1-25-43	Control			19	4.8	4.0	1
2	1-15-43	Benzene	11	-1				
4	1-25-43	Benzene						
5	1-8-43	Control	12	-20				
7	1-30-43	Control			12	4.1	2.9	1
6	1-8-43	Alcohol	12	-8				
8	1-30-43	Alcohol						
9	11-27-42	Control	6	-16				
10	11-27-42	pH 7	5	-14	2	5.3	0.38	
11	11-27-42	Control	11	-17				
13	5-21-43	Control						
12	11-27-42	pH 5.5	12	-3				
14	5-21-43	pH 5.5			14	4.8	2.9	1
15	12-4-42	Control	6	-10				
16	12-4-42	pH 9.2	5	+9	19	5.0	3.8	1
17	1-30-43	Control	6	-16				
18	2-5-43	Alc. treated	6	-5	11	2.6	4.2	1
19	4-3-43	Control	6	-21				
20	4-3-43	U.V.L.	6	-1	20	5.9	3.4	1

Table 1 (concl.)

21	4-3-43	Control	6	-21	14	5.8	2.4	5
22	4-3-43	Urea treated	5	-7				
23	6-23-43	Control	12	-15	5	3.3	1.5	5
23	7-1-43	Control						
24	6-23-43	Urea treated	10	-10				
24	7-1-43	Urea treated						
25	6-5-43	Urea	6	-16		4.1		
26	2-19-43	Control	11	-15	3	3.8	0.79	
26	2-26-43	Control						
27	2-19-43	Collodion	12	-12				
27	2-26-43	Collodion						

compound.

Results of this work very strongly indicate that this hormone will stand boiling at pH 7.0 or 8.0 for 15 minutes at 100° C. but not at a pH of 5.5 and 9.2. The destruction of this hormone by boiling at pH 5.5 and 9.2 could possibly be explained by hydrolysis, but this is probably not the case, because at such slight acidity or alkalinity, appreciable hydrolysis is very rare. It is an established fact that hydrogen or hydroxyl ions will denaturize protein; this is probably the proper explanation for this reaction.

Results of this study show that this factor is destroyed by treating with alcohol and urea. The work on urea denaturation was carried out in the late spring and summer while temperatures were around 30 to 90 degrees F., and the maximum calcium lowering was much less than that desired for controls, experiment also shows that urea alone will lower blood calcium appreciably. It was necessary to inject some urea into the experimental birds, and much of the calcium lowering in these experimental birds can probably be attributed to the urea. Had it been possible to obtain greater calcium lowering in the controls, or if the urea could have removed from the experimental extract, no doubt this work would have been more conclusive.

Irradiation with ultra-violet light will activate many reactions, and many native proteins will be destroyed by this treatment. These results demonstrate that this hormone was denatured by treating with ultra-violet light.

The results obtained in this work, without a doubt, demonstrate that the calcium lowering hormone passes through a collodion membrane through which hemoglobin will not pass.

Although denaturation work was not as conclusive as was desired, especially with urea, in summarizing all of the above data on denaturation, sufficient separate runs were made to strongly indicate that this hormone is destroyed by the common denaturing agents. This would prove the factor to be a protein in nature; the fact that it passes through a collodion membrane proves it to be one of relatively small molecular size.

SUMMARY

1. A simple, normal saline extract of one half of a fowl adrenal, weighing approximately 35 mg will very effectively lower blood calcium in the laying hen when injected intramuscularly. The hormone responsible for this lowering can be preserved for at least five months if the glands are kept in a sharp freezer.

2. The hormone is soluble in normal saline solution; infoluble in benzene; insoluble in alcohol; passes through a collodion membrane; is not destroyed by boiling 15 minutes at pH 7 or 8; is destroyed by boiling at pH 5.5 and 9.2; and is denatured by absolute ethyl alcohol, urea and ultra-violet light.

3. This hormone is not a steroid or simple organic compound, but is a protein of relatively small molecular weight.

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