THE ESTROGENIC CONTENT OF FRESH EGG YOLK, HEN OVARIES AND HEN FOLLICLES; AND A STUDY OF ITS EFFECT ON THE BLOOD CALCIUM OF FOWL

by

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INTRODUCTION

The observation that there is an increase in the blood calcium of hens accompanying egg production has been followed by attempts to discover the cause for this rise. The endocrine basis has been a subject of frequent investigations resulting in varying reports concerning the influence of the estrogenic hormones. The administration of fresh egg yolk has resulted in an increase in the blood calcium of fowl; the cause was attributed to the estrogenic hormone present in the yolk. However, conflicting reports also exist regarding the presence of the hormone in egg yolk.

A twofold purpose constituted the motive for this research. First, to determine quantitatively the estrogenic material present in fresh egg yolk, in hen ovaries in which were embedded small follicles, in follicle contents (unlaid egg yolk), and in the walls of the follicles. Second, to determine whether the estrogenic material from this avian source would increase the blood calcium level in immature fowls. Then, by knowing the estrogenic content of egg yolk and observing the effect of concentrated ex-

tracts of avian estrogenic material, judgment could be made of the influence of estrogenic hormone on the blood calcium when fowls were given injections of egg yolk.

ACKNOWLEDGMENT

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REVIEW OF LITERATURE

Among the sources in which attempts have been made to demonstrate the presence of estrogenic material are the hen ovaries and egg yolk. Allen, Whittsett, Hardy, and

Kneibert (1) extracted hen ovary follicles and fresh egg yolk separately with 95 per cent alcohol for two to four days, evaporated the alcohol and dissolved the residue in mazola oil. They found the ovary fraction to produce estrus growth in the genital tract of spayed rats, but reported a negative result for the egg yolk. Only 18 yolks were used. Glimm and Wadehn (2) also were unable to detect the estrogenic hormone in egg yolk. They stirred ten egg yolks in alcohol, filtered the precipitate, dried it and extracted it with chloroform. The combined extracts did not stimulate the growth of the uteri in rabbits.

Serono and Montezemolo (3) found that an estrogenic substance became demonstrable in chicken eggs only as incubation of the egg progressed. On the other hand, evidence of its presence was shown by Fellner (4) who tested the lipoid fraction of egg yolk on rabbits. This was confirmed by Kopec and Greenwood (5). They injected five egg yolks into a poulard over a period of twenty days and noted a growth of feminine feathers nine days after the last injection.

It is indicated that estrogenic material is more evident in the follicles of hen ovaries than in the freshly laid egg yolk. However, no data for comparison have been

made available. The use of a larger amount of egg yolk with thorough extractions should aid in formulating a more definite conclusion regarding its presence in the yolk.

A resume of the literature for the experimental evidences to associate the blood calcium level with sex and reproduction has been made recently by Marlow and Koch (6).

Pronounced evidences are found in the aves. The increase in blood calcium of pigeons as they come into laying was first noted by Riddle and Reinhardt (7). Hughes, Titus, and Smits (8) reported a high blood calcium in hens in production and low calcium during periods of nonproduction.

Various results have been reported concerning the effect of estrogenic hormone on the blood calcium of mammals and of fowls. Mirvish and Bosman (9) found that a crude ovarian extract caused a 30 per cent lowering of serum calcium in rabbits 24 hours after injection. A drop in serum calcium was also noted by Reiss and Marx (10) following an ovarian hormone application.

Marlow and Koch (6) studied the effect of noncrystalline purified estrogenic products prepared from pregnancy urine and from hog ovaries on fowl, rats, and rab-

bits. They interpreted the effect on blood calcium as not significant. Later Huey and Marlow (11) reported similar results from the application of fractions eliminated during the preparation of purified ovary extracts on rabbits.

Levin and Smith (12) also doubt that estrone has any effect on the blood calcium level of mammals as they found rats, rabbits, and monkeys unaffected by estrone administration or by ovariectomy.

Quite contrary to the foregoing results, Riddle and Dotti (13) reported particularly theelin and dihydrotheelin benzoate of the estrogens to be agents to cause a rise in the serum blood calcium in mammals as well as in pigeons and doves.

Recently Altmann and Hutt (14) were able to demonstrate at least a 35 per cent rise in the blood calcium of fowls by injecting as much as 100 grams of whole egg yolk into the peritoneal cavities. They attributed this increase to the influence of estrogenic hormone present in the yolk.

A synthetic product showing strong estrogenic properties was tested by Zondek and Marx (15). By administering four milligrams of 4:4' dihydroxy-α-β-diethylstilboestrol daily for six days, they observed an increase from 11.2 mg. to 41.4 mg. calcium per 100 cc. of blood in cocks.

This is by far the largest dosage that is reported to have been administered as one rat unit is approximately .0004 mg.

A laying hen perhaps is not normally stimulated by such large amounts of estrogenic material. To estimate the amount that could possibly be of influence necessitates a quantitative study of the amount available through the sex organs. A possible procedure lies in making a complete extraction of the organs and subsequently identifying its presence quantitatively.

EXPERIMENTAL DATA

On Determining the Estrogenic Material in Hen Ovaries, in Hen Follicles and in Fresh Egg Yolk

Extractions were made of hen ovaries in which were embedded follicles of five to six millimeters in diameter, of the contents of hen follicles averaging eight to ten grams in weight, of the follicle membranes, and of fresh egg yolk from eggs which were laid the same day as the process of extraction was begun. The ovaries and follicles were contributed by the Omaha Cold Storage Company, Omaha,

Nebraska. They were received in a frozen state and were immediately placed in a freezing temperature where they remained until work on them was begun.

Preparation of Materials for Extraction. Eleven pounds of frozen ovaries were ground fine and covered with 95 per cent alcohol. This was allowed to stand over night and the alcohol was separated through a cheesecloth.

The surrounding membranes were separated from six and one-half pounds of frozen follicles. Upon slightly thawing the membrane was easily removed. This yielded 80 grams of membrane which was immediately placed in 95 per cent alcohol. The contents of the follicles were ground and the fine material was stirred in 95 per cent alcohol to precipitate the proteins. On the following day, the alcohol was removed through a cheesecloth and the coagulated material was ground again.

The yolk was separated from the egg white and was beaten thoroughly. It was then poured slowly into 95 per cent alcohol with stirring. The precipitated material was allowed to stand in the alcohol overnight. The alcohol was removed through a cheesecloth.

Process of Extraction. The procedure of extraction was essentially the one described by Marlow and Groetsema

(16) for the extraction of residual ovaries from hogs. Modifications were necessary due to the presence of large amounts of fats and cholesterol.

About one and one-half to two pounds of the material were placed in a muslin sack and subsequently into the extractor (Fig. 1).

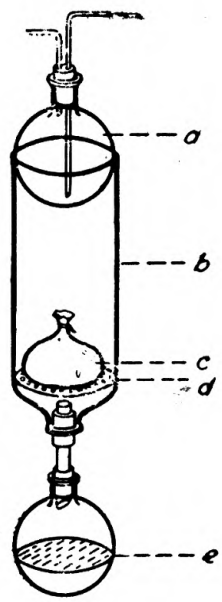

Fig. 1. Extractor. The vapors from the solvent in e go through the opening in the porcelain plate, d, and through the muslin sack, c, containing the ground material. The vapors are condensed on a and in the sack, and return to the receiver, e.

The solvent used in the first extraction was a combination of the alcohol which had previously covered the

ground materials and 95 per cent alcohol. The first extraction was terminated after several hours and the extract was replaced with fresh alcohol. Extraction continued until the returning condensed solvent was colorless and the material in the sack (yolk and follicles) was nearly free from pigments. The alcoholic extracts were combined and the extracted yolk and follicles served to make one cold ether extraction before they were discarded in a nearly white state. This step was eliminated from the process with the ovaries and the follicle walls. The ether extract was nearly evaporated and the residue was combined with the alcoholic extracts. The combined extraction liquors were allowed to stand over night in a slightly freezing temperature which hardened the fats and oils. The solution was siphoned from the fats and an equal volume of 95 per cent alcohol was added to them. This was warmed on a hot water bath with occasional shaking and the separation of the solution followed as before. The process of ablution was repeated once more.

These fats were also treated to extract any estrogenic material that might be present in combination. They were heated under a reflux condensor for several hours with an acidified alcoholic solvent. The alcohol contained 40 cc.

of concentrated HCl per liter. The liquor was separated by the chilling process which has been described. The fats were washed once with 95 per cent alcohol which was combined with the first extract. The solution was made neutral with alcoholic potassium hydroxide and the procedure from here followed as with the alcoholic extracts.

The alcoholic extract was evaporated under diminished pressure and the residue was taken up in a small volume of hot absolute alcohol. More fatty materials were frozen out, filtered by suction and washed with several portions of cold absolute alcohol. The filtrate was reduced down to dryness under diminished pressure and the residue was dissolved in ether. Three volumes of acetone were added and this was chilled to precipitate the phospholipins. The lipins were filtered from the solution by suction, washed with several portions of cold acetone, and the ether-acetone filtrate was evaporated to dryness under diminished pressure. The residue was dissolved in the least amount of hot absolute alcohol and allowed to stand in the ice box for at least overnight to crystallize cholesterol and separate any fatty material still present. These were filtered by suction, the filtrate was concentrated and the procedure of chilling repeated. After

several such cholesterol crystallizations, the final residue was dissolved in a measured volume of absolute alcohol and stored in the ice box.

The extract for injection was dissolved in olive oil by adding the oil to a portion of the alcoholic solution and then evaporating the alcohol under diminished pressure.

Biological Assay. The method of assay employed followed essentially the procedure devised by Kahnt and Doisy (17). The animals used were albino rats of the Wistar strain. They weighed about 200 gm. each.

Vaginal smears were made to determine the regularity of the estrus cycle. Those having regular cycles received a bilateral ovariectomy under ether anesthesia two weeks before use. Only those giving a diestrus smear for five consecutive days prior to use were kept.

An approximate estimation of the activity of the prepared extract was obtained before a rat unit was accurately determined. For this a series of single subcutaneous injections of varying amounts were made. To determine the rat unit an amount of the extract estimated to produce estrus in all rats was injected into each of five rats. The dose was administered in a series of three subcutaneous injections at four hour intervals. Injections of de-

creased dosages followed and the least amount able to produce full estrus in four out of five rats in 48 to 60 hours from the time of the first injection was considered to be one rat unit.

Estrus was determined by making vaginal smears with saliva moistened swabs of cotton. These were pressed out in a few drops of 0.9 per cent salt solution on a glass slide for observation under the low power of a microscope. The complete disappearance of leukocytes from the field and their replacement by squamous cells indicated estrus.

Results of the Assay. The results of the assay of the various purified extracts are shown in Table 1.

Table 1. Estrogenic activity of the extracts.

Source	Estrogenic activity
Fresh egg yolk	5 r.u./kilogram
HCl-alcohol extract following the alcohol extract	No activity
Hen follicle contents	44 r.u./kilogram
Hen follicle walls	166 r.u./kilogram of the membrane 6 r.u./kilogram on the basis of the weight of the follicles
Hen ovaries embedded with follicles 5 to 6 mm. in diameter	37 r.u./kilogram

On the Effect of Hen Follicles, Extracts of Hen Follicles and Extracts of Hen Ovaries on the Blood Calcium Level of Pullets

Since the foregoing results showed that the follicle contents (unlaid yolk) were over eight times as potent in estrogenic material as the same weight of fresh egg yolk, they served to be a better avian source of estrogenic material than fresh egg yolk to make a study of its effect on the blood calcium level of fowl.

The experiments were performed with 12 to 14 week old pullets of mixed breeds. Experimental and control groups alike were kept in the same pen under identical conditions. The temperature remained within a range of 10 degrees, the diet was the same throughout the experiment and the birds were kept from as little disturbance as possible before the bleedings were made. All of the bleedings were made from the wing vein; they were in every case made soon after the noon hour. Control calcium values were secured before treatment as well as from the blood of untreated birds taken at the same time as the blood from the experimental birds. The calcium determinations were made on whole blood by the method devised by Wang (18). Duplicate determina-

tions were made in all cases.

Effect of Follicle Contents (Unlaid Yolk). The follicle membranes were separated from some frozen follicles of the same size as were extracted and assayed previously. Weighed amounts of the follicle contents were thoroughly emulsified with a sterile 0.9 per cent salt solution and injected into the peritoneal cavity of six pullets. A total of 42 grams containing nearly two rat units were administered to each over a period of 12 days with injections made every third day. Six control birds received no injections. Bleedings were taken two days prior to the first injection and on the third, sixth, tenth, thirteenth, and seventeenth days after the first injection. The results are shown in Table 2. An increase in calcium was noted only on and after the thirteenth day after the first injection. The maximum increase was 1.7 milligrams per 100 cc. of blood. By this time all of the injected birds exhibited physiological disturbances due to the volumes of unabsorbable follicle contents contained in the peritoneal cavities. The results were even fatal in a few instances.

Effect of Follicle Extract and of Ovary Extract. An concentrated purified extract should furnish a better cri-

terion for a study on the effect of avian estrogenic material on the blood calcium than the follicle itself. By the application a more concentrated form of the estrogenic material could be administered, yet the ill effects due to large volumes of unabsorbable material would be eliminated.

Each of six pullets was given daily intermuscular injections for nine days of the extract which was equivalent to about 30 grams of the follicles per injection. This amounted to 1.3 rat units. Each of another group of six pullets received the same treatment with the extract of hen ovaries. Each injection was equivalent to about 60 grams of ovaries containing 2.2 rat units. The extracts were administered in olive oil. Five control pullets received injections of olive oil alone.

Two bleedings were taken before the injection so that each bird might serve as a control to a certain extent. The birds were then bled on the second, fifth, seventh, ninth, twelfth, and fourteenth days after the first injection. Analysis showed the blood calcium level to be subject to fluctuation to a certain extent as was evidenced in the control group as well as in the experimental groups. The results, as shown in Table 3, did not indicate a significant change in the calcium level due to the in-

jection of either extract. In the follicle extract injected group the greatest average calcium increase exhibited over its own average control value was 0.5 milligram and over the average of the control group was 0.7 milligram per 100 cc. of blood. On the other hand, on one occasion, the average calcium of the injected group fell to 0.6 milligram below that of its own average control value, and 0.2 milligram below that of the control group.

In the ovary extract injected group the differences were not even so obvious.

Table 2. Effect of follicle contents on blood Ca of pullets.

Group	No.	Mg. Ca/100 cc. of blood: Days before injection	Days after first injection: 3	6	10	13	17	Min.	Max.
		2							
Injected	1	11.6	11.4	11.0	11.3	**	-	11.0	11.4
do.	2	9.8	10.6	11.4	10.9	12.2	12.5	10.6	12.5
do.	3	11.8	11.4	11.5	10.1	12.4	12.6	10.1	12.6
do.	4	11.4	12.2	11.6	11.9	***	-	11.6	12.2
do.	5	12.2	11.2	12.9	*	-	-	11.2	12.9
do.	6	11.4	11.8	11.8	10.9	14.2	14.1	10.9	14.2
Average		11.4	11.3	11.7	11.0	12.9	13.1		
Minimum		9.8	10.6	11.0	10.1	12.2	12.5		
Maximum		12.2	12.2	12.9	11.9	14.2	14.1		
Control	7	12.6	11.4	10.6	10.7	11.8	11.5	10.6	12.6
do.	8	11.4	11.2	10.3	10.1	11.8	12.0	10.1	12.0
do.	9	11.2	11.4	10.6	11.7	11.2	11.7	11.2	11.7
do.	10	-	11.6	10.6	11.5	11.2	11.7	11.2	11.7
do.	11	11.4	12.5	11.2	11.9	11.4	11.1	11.1	12.5
do.	12	9.6	11.0	9.3	9.5	10.5	10.6	9.3	11.0
Average		11.2	11.5	10.5	10.9	11.3	11.4		
Minimum		9.6	11.0	9.3	9.5	10.5	10.6		
Maximum		12.6	12.5	11.2	11.9	11.8	12.0		

* Died of peritonitis.
** Died. No autopsy was made as the bird was discarded by the caretaker.
*** The bird was sick and was killed for observation. The yolk was packed solidly against the organs and intestines. No bad odor was found as in the case of peritonitis.

Table 3. Effect of follicle and ovary extracts on blood Ca of pullets.

Group	No.	Mg. Ca/100 cc. of blood: Days before injection: 3	1	Days after first injection: 2	5	7	9	Days after injection ceased: 3	5	Min.	Max.
Follicle extract	1	12.1	11.3	12.5	12.2	12.2	10.8	12.4	12.0	10.8	12.5
do.	2	11.0	10.8	11.8	11.2	11.6	11.8	10.6	10.6	10.6	11.8
do.	3	11.2	10.3	12.5	12.2	11.6	11.5	11.6	10.3	10.3	12.5
do.	4	12.7	12.1	13.1	12.6	12.6	11.5	13.2	12.4	13.2	12.4
do.	5	12.2	11.7	11.6	11.0	11.8	11.2	11.0	11.2	11.0	11.8
do.	6	11.6	11.9	12.7	11.8	12.0	10.7	11.8	12.3	10.7	12.7
Average		11.8	11.4	12.3	11.5	11.6	11.2	11.8	11.5		
Minimum		11.0	10.3	11.5	11.0	11.6	10.7	10.6	10.3		
Maximum		12.7	12.1	13.1	12.6	12.6	11.8	13.2	12.4		
Ovary extract	7	11.4	12.1	12.2	12.2	11.8	11.5	11.5	11.2	11.2	12.2
do.	8	12.4	12.1	12.7	12.6	12.8	11.5	12.2	12.0	11.5	12.8
do.	9	12.0	11.3	12.2	11.8	11.4	11.2	12.2	12.4	11.2	12.4
do.	10	11.8	11.0	11.4	11.2	11.2	11.0	11.0	11.8	11.0	11.8
do.	11	12.7	10.6	12.0	12.0	11.8	11.5	11.6	12.2	11.5	12.2
do.	12	11.8	11.6	12.4	11.8	12.2	12.9	11.8	12.4	11.8	12.9
Average		12.0	11.3	12.1	11.9	11.8	11.6	11.7	12.0		
Minimum		11.4	10.6	11.4	11.2	11.2	11.0	11.0	11.2		
Maximum		12.7	12.1	12.7	12.6	12.8	12.9	12.2	12.4		
Control	13	11.4	11.9	11.6	11.0	11.2	10.0	12.0	10.6	10.0	12.0
do.	14	12.9	12.9	13.1	12.6	12.0	11.8	12.0	-	11.8	13.1
do.	15	11.6	11.6	11.9	12.6	12.0	11.5	11.5	11.5	11.5	12.6
do.	16	11.9	11.3	10.4	10.6	11.4	11.0	11.2	11.4	10.4	11.9
do.	17	-	11.1	11.2	11.4	11.4	10.6	13.1	11.4	10.6	13.1
Average		11.9	11.7	11.6	11.6	11.5	11.0	12.0	11.2		
Minimum		11.4	11.1	11.2	10.6	11.2	10.0	11.2	10.6		
Maximum		12.9	12.9	13.1	12.6	12.0	11.8	13.1	11.5		

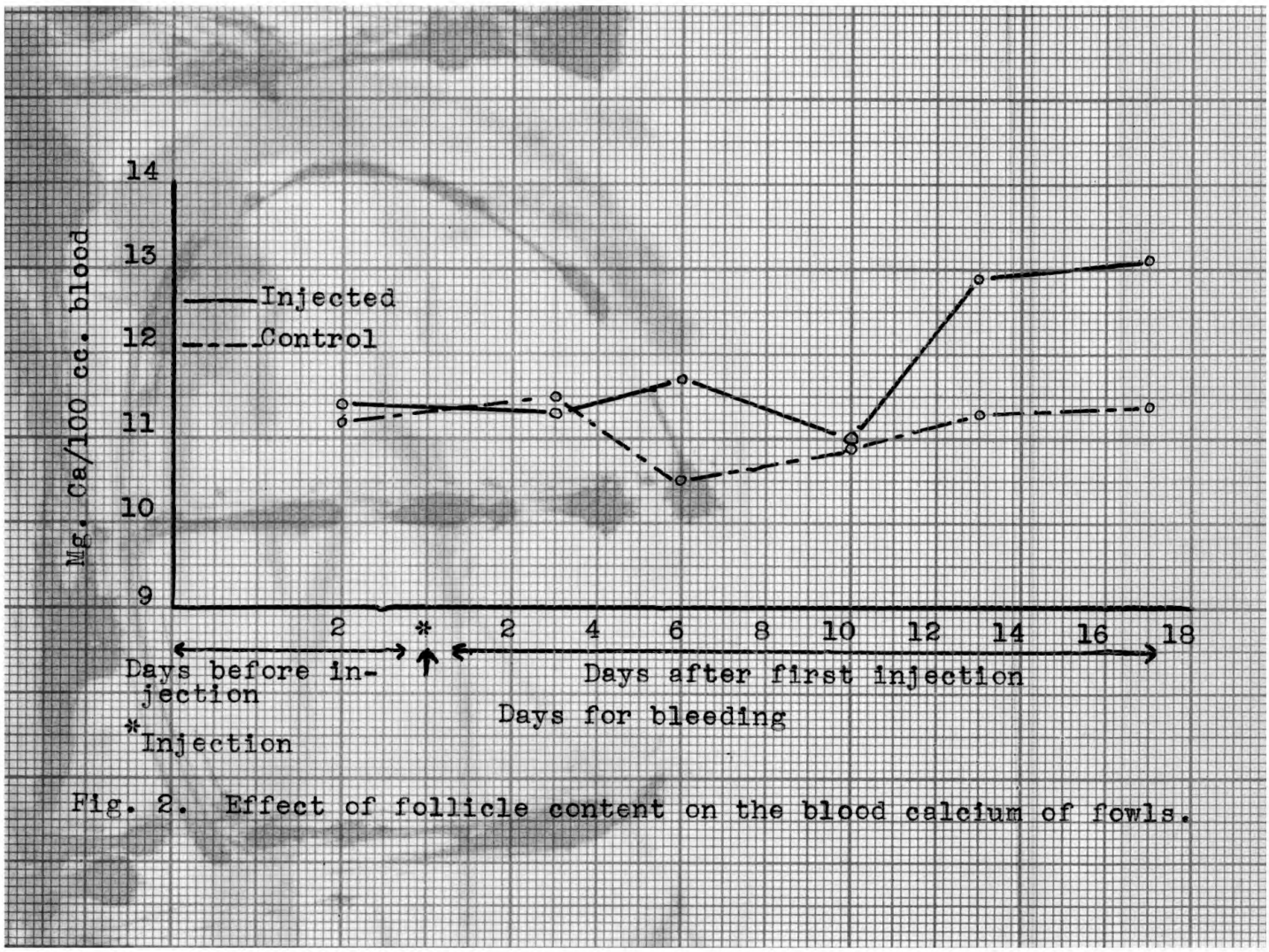

Fig. 2. Effect of follicle content on the blood calcium of fowls.

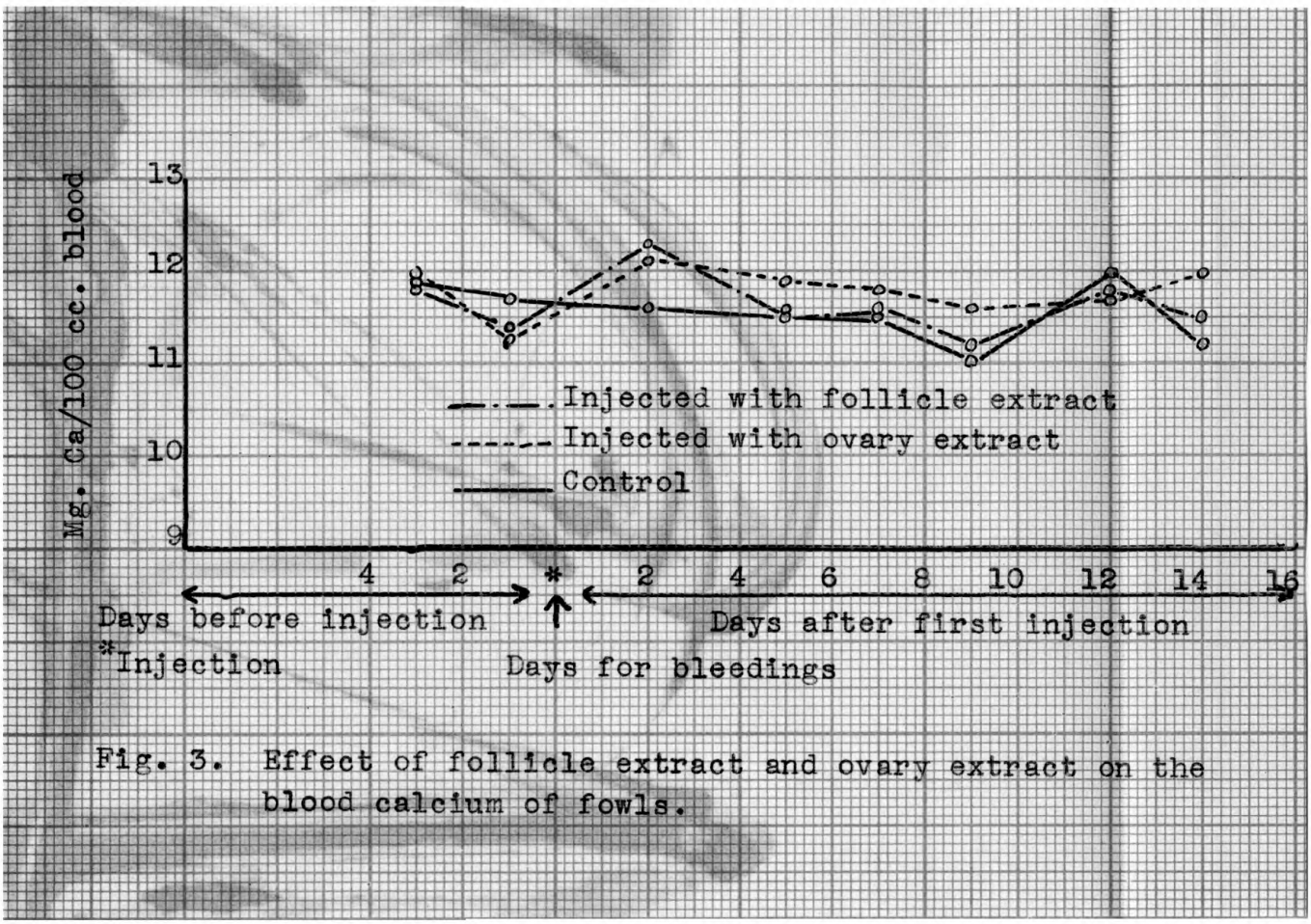

Fig. 3. Effect of follicle extract and ovary extract on the blood calcium of fowls.

DISCUSSION

The results from this research have a bearing on two subjects. First, on the estrogenic content of hen ovaries, of hen follicles, and of fresh egg yolk; and second, on the effect of the estrogenic material on the blood calcium level of fowls.

Estrogenic material was found to be present in egg yolk in small amounts. The small value perhaps indicates the necessity of using larger amounts of the yolk to identify its presence than was used by those who have reported negative results.

The follicle contents (unlaid yolk) contained over eight times as much estrogenic material per unit weight as the fresh egg yolk. This provided a better source of estrogenic material than the fresh egg yolk for a study of its effect on the blood calcium of pullets. The administration of 42 grams of the whole material, following the procedure of Altmann and Hutt (14), who used fresh egg yolk, had no appreciable effect. An increase of 1.7 milligrams per 100 cc. of blood was noted only after the birds exhibited marked physiological disturbances due to the volume of

unabsorbable follicle contents contained in the peritoneal cavities.

The concentrated extracts of the follicles and of the ovaries produced no physiological disturbances. The extract of the follicles that was administered to each bird was equivalent to 270 grams of the unlaid egg yolk, and contained over 20 times the amount of estrogenic material that Altmann and Hutt (14) administered through the fresh egg yolk. Since this produced no appreciable change in the blood calcium, it seems doubtful that the one-half rat unit of estrogenic material contained in 100 grams of yolk would have an appreciable influence on the blood calcium.

The results of the study of the estrogenic content of the ovaries and the follicles indicate that the amount present in a laying hen is small. The average weight of the ovaries and follicles in a laying hen is less than 100 grams. Then on the basis of the findings that the follicles contain about 50 rat units per kilogram and the ovaries with immature follicles contain about 37 rat units per kilogram, the total amount in the hen is less than five rat units. However, this statement is based on the assumption that larger amounts of the estrogenic material are not in other parts of the body. Investigation is necessary and

is in progress to throw more light on the problem.

SUMMARY

1. The yolk from freshly laid eggs contained five rat units of estrogenic material per kilogram of yolk.

2. The follicle contents of hen ovaries contained 44 rat units of estrogenic material per kilogram of follicles.

3. The follicle walls contained six rat units of estrogenic material per kilogram of follicles.

4. The hen ovaries in which were embedded small follicles of five to six millimeters in diameter contained 37 rat units of estrogenic material per kilogram of ovaries.

5. An concentrated extract of hen ovaries and of hen ovary follicles did not produce a significant change in the blood calcium level of pullets.

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