THE CALCIUM AND PHOSPHORUS METABOLISM IN THE BLOOD OF THE LAYING HEN AS RELATED TO EGG-SHELL FORMATION

by

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B. S., Brooklyn College, 1933

A THESIS

submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1936

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INTRODUCTION AND REVIEW OF LITERATURE

Owing to the nature and extent of the field covered by this research it was believed advisable to consult and review as many papers as possible regarding not only the work actually carried out but also all phases of calcium and phosphorus metabolism that may possibly play a part in egg shell formation. Though the papers cited below are by no means a complete bibliography on the subject, they give, in the estimation of the author, a fair representation of the work in the field.

The metabolism of calcium in the animal body has long been the subject of a great deal of scientific investigation. Though the early workers and many of the more recent papers cited were more concerned with mammalian blood and not directly with the blood of the hen, the results obtained serve to clarify many of the problems which arise in the study of calcium metabolism in the laying hen.

Thus, as early as 1909 Mendel and Benedict (1) reported that the mammalian body is able to retain for some time a considerable quantity of excess calcium which may be introduced. This is of considerable interest since one of the first questions that arises in the study of the use of

calcium by the hen for shell formation is whether the hen can store in her body a supply of calcium upon which she can later draw as the need arises. This question was not attacked directly until many years later when the work of Halnan (2) indicated that the hen might be storing a reserve of calcium during the period of non-production upon which she could later draw when shell formation created a large demand for calcium. Halnan reported a storage of calcium which occurs only a short period before laying. He stated that the extra demand for calcium during egg laying was met by increased retention from the feed. Russell and McDonald (3) also reported a positive calcium balance in the hen during non-production periods.

Common (4) carried the investigation still further and by complete studies on the calcium consumption and excretion in hens found that there is a 20 to 75 per cent rise in calcium retention during a two week period before the onset of laying. In a later paper (5) he states: "The $CaO:P_2O_5$ retention in the 2-3 week storage period before laying is substantially that of $Ca_3(PO_4)_2$. It therefore seems plausible that the reserve is being laid down principally as bone."

Working with pigeons, Kyes and Potter (6) found that whereas the inner surface of the shaft of the femurs of male and non-laying pigeons were smooth and devoid of bony spicules

and the marrow did not show any ossification, as the bird approached the laying condition, as determined by the size of the ova present, ossification of the marrow set in and the inner surface of the shaft of the femur became rough with bony spicules. Extreme ossification was found only in those birds with the largest ova (9.1 mm.). This would indicate that the pigeon was storing calcium preparatory to the onset of laying.

This view was further substantiated when Deobald, Lease, Hart, and Halpin (7) found that hens on a high calcium ration were capable of storing the excess calcium in their bones so that the ash content of their bones was approximately 10 per cent higher on the average than the ash content of the bones of hens on a low calcium ration. They concluded from this that about 10 per cent of the calcium stored in the bones could be withdrawn for the purpose of egg-shell formation. That this is actually the case is indicated by their further finding that removal of calcium from the laying hen's diet caused a diminution in the ash content of the shell and finally a complete cessation of egg laying. This would indicate that at first the hen is able to draw on her reserve calcium for shell formation but when this reserve is depleted egg-laying is forced to cease.

If, then, the calcium is being retained in the form of bone there should be a similar, simultaneous retention of phosphorus. That such is actually the case was shown by several of the workers cited above. Halnan (2) found a storage of phosphorus a short period before laying began. Russell and McDonald (3) also found a positive phosphorus balance during non-production, but stated that the percentage of the phosphorus retained was greater than that of the calcium. As pointed out above, however, Common (5) in more recent work found the $CaO:P_2O_5$ retention during the 2-3 week period before laying to be substantially that of $Ca_3(PO_4)_2$.

Then, if the hen is to obtain the calcium for shell deposition from a bone reserve, one might be led to suspect that this fact might be reflected in the serum phosphatase level of the blood of laying hens. Working on this possibility, Common (8) demonstrated that the phosphatase in cockerels averaged about 4.0 units, whereas that for laying hens averaged about 15.0 units. The level in one pullet nearing laying was found to be 12.1 units. He concluded that "the values for cockerels and sexually immature pullets are comparable, those for laying and moulting birds are higher." This would indicate the presence of a mechanism in laying hens for the solution of bone and the splitting of $Ca_3(PO_4)_2$ for the probable purpose of liberating calcium and

making it available to be laid down as calcium carbonate in the shell.

Passing from the storage of calcium in the form of bone, which is neither an unreasonable nor a startling procedure, one encounters in the study of the calcium level of the blood of laying hens an entirely unsuspected and highly unusual phase of calcium metabolism--namely, a tremendous rise in the serum calcium content in the laying bird to 2-3 times the level in non-laying birds. In 1926 Riddle and Reinhardt (9) found that "the advent of ovulation in the pigeon was preceded by a rise of blood-calcium in the female 123 hours before the beginning of the formation of an egg-shell." However, they believed that this was in no way explained by "a large calcium 'need' for shell formation," but was, rather, an expression of increased parathyroid activity.

Hughes, Titus, and Smits (10) working with hens in 1927 reported that "the calcium content of the blood of hens during the period of egg production is about double that during the period of non-production." Heller, Paul, and Thompson (11), studying the life cycle of the chicken, found that calcium increased greatly during egg production and returned to former lower levels at molting time.

Inasmuch as it seemed physiologically impossible for the hen to tolerate such a large increase in an ionized form of calcium, Correll (12) studied the filterability of the excess calcium through a "Cellophane" membrane, and found that the increase in calcium could be entirely accounted for by an increase in the non-filterable fraction. In other words, though the calcium rose greatly in the hen's blood there was no change in the amount of the unionized form. Heller, et al (11) in the following year investigated further the nature of the calcium rise and found the increase to be represented in both the non-diffusible fractions--the protein-bound calcium and the non-filterable adsorbable calcium. This confirmed the work of Benjamin and Hess (13), which will be discussed more fully below.

At this point it is advisable to review the literature pertaining to the diffusibility of the calcium in serum, but before doing so it would be well to outline briefly the forms of calcium and inorganic phosphorus found in serum as reported by Benjamin and Hess (13). They found that the total calcium could be divided into four fractions:

1. a filterable, adsorbable calcium-phosphorus complex;
2. a filterable, non-adsorbable fraction containing the ionized calcium;
3. a non-filterable, adsorbable fraction; and

4. a remainder, neither filterable nor adsorbable, usually described as "protein-bound" calcium.

The differentiation between adsorbable and non-adsorbable varieties of calcium was based on the fact that when serum and ultrafiltrate are shaken with $BaSO_4$, differing amounts of calcium are adsorbed from each. In a previous paper (14) the inorganic phosphorus, which is normally completely filterable in mammalian serum, is divided into:

1. a filterable, adsorbable calcium-phosphorus complex; and

2. a filterable remainder which is the phosphate ion.

However, in hypercalcemia, such as exists in the laying hen, a third fraction, a non-filterable adsorbable calcium-phosphorus complex, is known to exist. Grollman (15) concluded from his work that the inorganic phosphorus must be present in blood not only "as a simple, diffusible phosphate anion, but that it may also be present in the form of some non-diffusible complex. The dependence of the diffusible phosphorus on the calcium concentration would indicate that this complex is either a colloidal combination of calcium and phosphate stabilized by the blood proteins-------or in actual combination with them-------".

Heller, et al (11) recently confirmed this non-filterable adsorbable complex but found that it "is not present in

significant quantities in young hens, but makes its appearance about 8 weeks before egg production begins" and then rises rapidly until it reaches about 8 mg. at the time of production.

Thus it has been known for some time that calcium and inorganic phosphorus exist in the blood in at least two forms--diffusible and non-diffusible. Studies of the two forms of calcium early revealed that whereas the diffusible form of calcium is rather constant in body fluids, the non-diffusible calcium may vary greatly.

Salvesen and Linder (16) reported that it was the uncombined calcium that is kept constant in body fluids. Updegraff, Greenberg, and Clark (17) confirmed the earlier work and further found that the non-diffusible calcium is not in equilibrium with the total diffusible calcium.

The manner in which the non-diffusible calcium was held in the serum was the subject of much experiment, the results of which led early workers to the conclusion that this form of calcium could be completely accounted for as bound to the serum proteins. Marrack and Thacker (18) concluded that calcium forms an unionized compound with proteins, the formation of this compound accounting for the non-diffusible fraction of the calcium. Updegraff, et al (17) presented further evidence indicating that the non-diffusible calcium is bound

to the serum proteins in an unionized form. Studying the effect of pure protein solutions and blood serum on the diffusibility of calcium, Loeb (19) believed he had demonstrated that the diffusibility of calcium from blood serum and from solutions of pure proteins is essentially the same process and governed by the same factors, i.e., the Donnan equilibrium effect and the formation of complex ions between calcium and the protein. He concluded by saying, "The behavior of calcium finds its explanation in the Donnan theory of membrane equilibria slightly modified by the formation of complex calcium-protein ions."

However, more recent work has brought forth the fact that the non-diffusible calcium is present in at least two forms--one being the protein-bound calcium, which is non-adsorbable on $BaSO_4$, and the other being an adsorbable form of non-diffusible calcium. This fact was brought forth by Benjamin and Hess (13) in 1933. The same year Laskowski (20) stated that there was present a calcium phosphate complex with protein. The following year Smith (21) showed that when excess calcium was added to serum part of it became non-diffusible, part of this non-diffusible calcium being in the form of $Ca_3(PO_4)_2$. He stated that the binding of the calcium by the protein and the formation of the insoluble $Ca_3(PO_4)_2$ went on simultaneously.

Benjamin and Hess (13) investigated the hypercalcemia of laying hens and found that the rise in calcium as the hen went into production was due almost entirely to a rise in the non-diffusible calcium. This is in agreement with the work of Correll (12) previously cited. Benjamin and Hess (13) found further that the rise was about equally divided between the adsorbable and non-adsorbable calcium. They attribute the rise in the non-adsorbable fraction to an approximately 50 per cent increase in the serum proteins of the laying hen. Taylor and Russell (22) confirmed the fact that the rise in calcium in the laying hen was confined wholly to the non-diffusible part of the calcium.

From the above it can be seen that there is a close interrelationship between the calcium and phosphorus in the serum and that they mutually affect the diffusibility of each other. Grollman (15) found that the inorganic phosphorus in mammalian serum gradually became non-diffusible as the serum calcium was increased. This work was confirmed by Benjamin and Hess (14), and Laskowski (20). Smith's work (21) has been given above, his results showing that as the calcium in the serum increased there was a definite partition of the fraction which became non-diffusible between a protein bound form and a Ca:P complex form. Further, both these forms were produced simultaneously and the Ca:P complex was shown to be

$Ca_3(PO_4)_2$.

Leaving this more general discussion of calcium and phosphorus metabolism, let us now review the work done directly in regard to egg production in the hen. Several workers have investigated the rate of calcium absorption from the digestive tract of the laying hen with the idea of learning whether the calcium for the egg shell is absorbed from the intestine and carried directly to the shell gland during the period of shell deposition. Buckner, Martin, and Hull (23) drew blood from the anterior mesenteric artery and vein of the laying and non-laying hen and demonstrated that whereas there were 2.5 mg. more calcium in the vein than in the artery of the laying hen, the calcium content of the vein and artery of the non-laying hen was the same. This would indicate an active absorption of calcium from the intestine of the laying hen, presumably for the purpose of shell formation. Common (5, 24) also leans toward the view that if the hen receives sufficient calcium in its daily diet it does not have to draw on its body reserve of stored calcium.

That a readily available source of calcium is always essential to the laying bird was shown by Buckner, Martin, and Insko, Jr. (25). They deprived laying birds of calcium and found that there was a marked decrease in egg production

and in the calcium carbonate in the shell in 2-4 weeks, indicating that the hen had a reserve store which was more or less completely exhausted in 2-4 weeks. Deobald, Lease, Hart, and Halpin (7) also found that very thin-shelled eggs, containing only 25 per cent of the normal ash content, were produced by suddenly removing the calcium from the ration. These workers found that there was a gradual diminution of the CO_2-free ash of the shell, with a virtual cessation of egg production by as early as the twelfth day after the removal of the calcium.

The question then arises: How is the calcium carried to the shell and which of the several forms of blood calcium is the one concerned with calcification? That the calcium is transported to the shell gland by the uterine artery and then removed as needed during deposition was shown by Knowles, Hart, and Halpin (26). They succeeded in obtaining one complete sample of blood from the uterine artery and uterine vein during the time of shell deposition. Their analysis of this blood revealed an excess of 7.7 mg. of calcium per 100 cc. of blood in the artery over the vein. This shows an active removal of calcium from the blood stream by the shell gland during the period of egg-shell formation.

Benjamin (27) first investigated the different forms of calcium and their relation to calcification. Working with

serum and with artificial solutions of the inorganic elements of serum, she found that "normal calcification of bone is brought about by a process of adsorption of the calcium-phosphorus complex and that this form, and not ionic or other forms of calcium, is the type primarily involved in calcification." The calcium-phosphorus complex referred to is the filterable, adsorbable calcium-phosphorus complex, and the calcification is that of bone.

Investigating the hypercalcemia of laying hens Benjamin and Hess (13) remark, "It is of interest that the hypercalcemia of laying hens, which is presumably associated with egg production, fails to involve an increase in the amount of the filterable calcium-phosphorus complex. This form of calcium has been found to be the one primarily concerned in calcification of bone." Working on this phase of calcium metabolism in the hen, Heller, et al (11) report, "-----it appears that the non-filterable, adsorbable complex form of calcium is most closely associated with the process of egg-shell formation and it must be the fraction which is physiologically active and therefore responsible directly or indirectly for the larger portion of calcium used in egg production."

A method of attack which has proved very valuable in studying the problem of the mechanism of egg production is

analysis of the droppings of laying and non-laying hens. These analyses brought to light the interesting fact that during the period of egg production the phosphorus excretion in the droppings increased greatly, the calcium to phosphorus ratio decreasing, as studied by Halnan (2). The latter stated that in non-laying pullets the Ca:P ratio was as in $Ca_3(PO_4)_2$, but this was shown to be dependent upon the Ca:P ratio in the diet by Russell and McDonald (3) and by Common (4). The former workers further showed that "with high production there may be periods of negative phosphorus balance," and that the phosphorus in the excreta during the production period is greater than that necessary to form tricalcium phosphate with the excreted calcium.

Common conducted an extensive investigation of the excreta of laying and non-laying hens and published his findings in a series of papers. He reported (4) a heavy excretion of phosphorus during egg laying, saying "The amount voided in this way exceeding that voided in the egg." Further, the heavy phosphorus excretion occurred about a day or so before the laying of the egg, corresponding with the period of shell formation. He also found that the heavy phosphorus excretion did not involve a concurrent heavy excretion of calcium.

He also reported (5) the excess phosphorus excreted to be water soluble and suggested a urinary route, while in (24) he found the heavy phosphorus excretion was accompanied by a simultaneous increase in ammonia nitrogen, indicating that the excess phosphorus is excreted in the urine as ammonium phosphate. This is in contrast to the conditions in non-laying hens as reported by Knowles, Watkin, and Hendry (28), who found but traces of a water-soluble phosphate in the excreta of non-laying hens. They conclude that the phosphate is present as dicalcium phosphate. In (24) Common further shows that the heavy phosphorus excretion does not accompany egg laying provided the calcium carbonate intake is sufficiently high. The significance of these results will be discussed later.

There is yet one more phase of the changes which take place in the hen's blood with the advent of maturity and productivity. This is the appearance in the serum of a phosphoprotein as demonstrated independently by Roepke and Hughes (29) and Laskowski (30). Roepke and Bushnell (31) showed this phosphoprotein to be apparently chemically and immunologically identical with the vitellin of the egg yolk. This phosphoprotein is present in but small amounts, if at all, in the serum of males or non-laying females, but is quite appreciable in amount in the blood of laying hens.

The purpose of the present investigation was to attempt to throw further light upon the manner in which the hen utilizes calcium for the purpose of laying down calcium carbonate in the form of an egg shell. Four avenues of approach were utilized in attempting to solve this problem. Thus the calcium and protein phosphorus in relation to the production cycle, the calcium and phosphorus level in relation to the single egg cycle, the ultrafilterability of the inorganic phosphorus, and the calcium and phosphorus content of the leg (ischiatic) artery and vein were studied.

PROCEDURE

Method of Drawing and Handling Blood

In all cases with the exception of the leg (ischiatic) bleedings the blood was drawn from the wing vein. The feathers on the under side of the wing along the forearm are plucked, the skin directly over the vein is drawn to one side of the vein, and an incision parallel to the vein is made with a sharp scalpel. The skin is then allowed to return to place, whereupon the incision, if it has been correctly made, lies directly over the vein. The vein is thus exposed as a large, prominent blood vessel and is easily entered with a hypodermic needle.

A sharp needle of 22 gauge is then carefully inserted into the vein in a direction opposite to the flow of blood, i.e., in a direction toward the distal end of the wing. Blood is then withdrawn with a 5 cc. syringe, care being taken to draw the blood slowly so as not to collapse the vein.

Before withdrawing the needle from the vein the thumb is placed distal to the point of the needle and held there for a few minutes after the needle is withdrawn, at which time the puncture has clotted and little if any hemorrhage is obtained. If several bleedings are to be made a long skin incision is advisable, running the whole length of the forearm. At succeeding bleedings any clot that has formed is wiped away with a moist cloth, and the vein is usually found in excellent condition. As many as 6 successive bleedings have been made from a single vein in a period of 24 hours.

This method of drawing blood has been found very satisfactory, but its success depends greatly upon the use of a capable assistant for the restraint of the bird. The bird is laid on its side, the assistant grasps the legs of the bird with one hand and the head and upper wing with the other and extends the bird. The wing is held open for the bleeding, inner side up and parallel to the table. Since the hen is extremely resistant to infection no antiseptic precautions

are necessary.

In those cases where but a single bleeding was made, 5 cc. of blood were collected. Where a number of bleedings were to be made in 24 hours, only 2 cc. of blood were taken at each bleeding.

Wherever feasible the blood was treated with an anticoagulant (as indicated) and the plasma collected by centrifuging and decanting. The reason for this was that the yield of serum obtained by allowing the blood to clot was never more than 50 per cent and the volume of serum thus obtained was often too small to work with, whereas the yield of plasma was about 70 per cent.

The most convenient method for preparing the tubes with anticoagulant (sodium oxalate or citrate) was found to be that of using a 2 per cent solution of the salt, introducing about 0.15 cc. of the solution per 1 cc. of blood to be collected into the tubes, and evaporating them in a nearly horizontal position on a hot-plate. This gave a thin coating of the anticoagulant which easily dissolved in the blood on shaking. In citrating tubes the tubes must be carefully watched and removed from the hot-plate as soon as dry since this substance chars easily.

Ultrafiltration

The method of ultrafiltration used was essentially the same as that described by Nicholas (32) and Correll (12). A filter similar to the one used by the latter, but smaller in size, was employed. The #300 "Cellophane" was kindly supplied by the Du Pont Cellophane Co., Inc. A pressure of 140 to 180 pounds was obtained by means of a compressed nitrogen tank.

The filter employed had a membrane area of only about 8 sq. cm. so that ultrafiltration of the serum was very slow, about 2 to 3 hours being required for the collection of little more than 0.5 cc. of ultrafiltrate.

Determination of Protein Phosphorus

The protein phosphorus was determined by the method outlined by Roepke (33) with several slight modifications incident to the use of but 1 cc. of serum. Further, the final color was produced by reduction with 0.25 per cent alpha-amino-naphthol-sulfonic acid instead of stannous chloride.

The method consists essentially in the precipitation of the plasma proteins with 10 per cent trichloracetic acid,

washing several times with 10 per cent trichloracetic acid and then several times with a hot alcohol-ether mixture to remove the lipoids. The protein is then ashed with a 50 per cent magnesium nitrate solution, the ash dissolved in 10 N. sulfuric acid, the solution neutralized to congo red paper with concentrated ammonium hydroxide, diluted to the desired volume, and the color developed and compared with a suitable phosphorus standard.

It was found advisable to use alpha-amino-naphthol-sulfonic acid as the reducing agent since the color so developed does not fade as does the one obtained with stannous chloride.

Calcium and Inorganic Phosphorus Determination

The calcium determination was made in accordance with the method of Fiske and Subbarrow (34) on either serum or citrated plasma. The calcium is precipitated as the phosphate, washed with an alkaline alcoholic wash water, dissolved in a 2.5 per cent ammonium molybdate in 3 N. H_2SO_4 solution, reduced with 0.25 per cent alpha-amino-naphthol sulfonic acid and the color compared with a suitable standard in a colorimeter.

The inorganic phosphorus was also determined by the method of Fiske and Subbarrow (34), serum or oxalated plasma being used in this case.

Operation for Drawing Blood from Ischiatic Artery and Vein

The bird is placed under Nembutal anesthesia, 3/4 cc. of Nembutal being injected into the wing vein, and the region over the upper leg is plucked free of feathers. The femur is palpated through the skin and is used as a landmark. With a sharp scalpel a 2-3" incision is made directly above and along the line of the femur, starting about 1½-2" from the distal end of the femur and extending the incision dorsally. This exposes the line of junction of the two parts of the gluteus primus muscle.

Using a blunt probe, and a scalpel where necessary, the posterior part of the gluteus primus is carefully separated from the anterior part. When the separation is complete a retractor is attached to the posterior part of the muscle to keep the entrance open. At the base of the opening is encountered some intermuscular fascia. This is easily ripped through with a blunt probe, exposing the ischiatic blood vessels and nerve lying side by side in a groove between the vastus externus muscle anteriorly and the biceps flexor cruris muscle posteriorly. The vein is seen as a large, purplish-blue vessel lying most anteriorly; the nerve is a flattened, glistening cord which lies posteriorly; and the

artery is a small, round, white vessel lying in the middle.

In handling the vessels it has been found most convenient to use a pair of fine-pointed, rounded thumb forceps. The wall of the upper end of the vein is grasped with the forceps and the vein is easily lifted to within working distance. A 26 gauge needle on a 2 cc. hypodermic syringe is inserted into the vein in a distal direction and the blood drawn. Before withdrawing the needle the vein is grasped below the point of the needle with a pair of forceps, and is held clamped for 2-3 minutes after the needle is withdrawn, allowing the puncture to clot and preventing hemorrhage. The vein is then allowed to return to place.

The lower end of the artery is then grasped with the forceps and the artery lifted out. The needle is inserted in a proximal direction this time and the blood drawn. The same precautions against hemorrhage are again observed and the artery returned to place.

The muscles are allowed to come back to their normal position, and two or three skin sutures are taken as needed. If the operation has been performed carefully and efficiently there has been little if any cutting into of muscles, slight and easily controlled hemorrhage, and the hen has suffered no inconvenience. Recovery is normally immediate and complete, the hen showing little or no trace of a limp

upon coming out of the anesthetic.

The success of the operation depends upon a thorough acquaintanceship with the anatomy of the region, efficient lighting, care in separating the muscles and handling the vessels, and a capable assistant for the handling of the forceps while the operator draws the blood.

The retractor mentioned above was set up very simply but with excellent results by using a bent paper clip at the end of a strong rubber band which is attached to a ring stand. The amount of traction can be excellently controlled by regulating the distance of the ring stand from the field of operation.

Animals Used

In studying the relationship between the protein phosphorus and rise in calcium, ten immature pullets were tested at intervals until they came into production. Five cc. of blood was drawn from the wing vein at each bleeding and citrated, the plasma being used for the analysis.

For the study of the variation in the blood calcium and phosphorus in a 26 hour period during the single egg cycle in the laying hen and during a corresponding period in the non-laying hen, 10 of the former and 5 of the latter were used. Hens were chosen that laid within a period of one

hour, as found by trapping, and then bled together at the intervals shown in Table 3. Three or four hens were usually run at a time. Serum was used for the analysis on laying hens A to G inclusive and on non-laying hen V. Oxalated plasma was used from laying hens H, I, J, and non-laying hens W, X, Y, Z.

The blood obtained was treated as necessary immediately upon drawing, and the serum or plasma collected. The latter was then immediately treated with 10% trichloracetic acid and centrifuged and the filtrate placed in a cooler. When all the bleedings from one set of hens were completed (at the end of the 26 hour period) the complete set of filtrates from each hen was analyzed together and compared with the same standard. In this way all possible error was reduced to a minimum.

In the ultrafiltrate experiment three hens were used and the blood collected at a low and high phosphorus level, each hen being bled once at each stage. The serum was used from all hens in the experiment. The serum was kept in a cooler, and analysis on the serum was made together with the analysis of the corresponding ultrafiltrate to eliminate any possible effect of difference in the standing time.

At the start of the experiment involving the drawing of blood from the ischiatic vessels, it was thought advisable

Table 1

Total Calcium and Protein Phosphorus in Plasma (Mg. per 100 cc.).

Hen No.	11-16-35 Total Ca	11-16-35 Protein P	11-23-35 Total Ca	11-23-35 Protein P	12-7-35 Total Ca	12-7-35 Protein P	12-14-35 Total Ca	12-14-35 Protein P	12-21-35 Total Ca	12-21-35 Protein P	12-28-35 Total Ca	12-28-35 Protein P	1-11-36 Total Ca	1-11-36 Protein P
1420			12.95	absent			23.26	7.27			25.96	12.17		
1421	12.37	trace	12.95	trace			16.04	1.49	11.49	faint trace			12.20	trace
1422	22.29	6.94			19.70				20.84	9.75			22.22	10.31
1423			14.23	0.65			23.00	6.13					18.92	6.43
1424	11.52	absent	13.12	trace	20.40				22.72	10.46			18.44	7.84
1425	13.01	trace	13.13	0.40			26.66	12.22					19.52	7.05
1426	12.83	absent	12.90	absent			14.42	trace			19.60	7.33	17.78	6.11
1427	20.05	5.41			20.40				12.03	trace				
1428	13.29	0.66	13.23	trace			16.30	heavy trace	13.75	1.57			20.52	7.27
1429	13.23	trace	13.34	trace			14.50	trace	11.32	trace			11.72	absent

to use a bird but once owing to the extent of the operation. Therefore one bird was bled when the egg was in the upper part of the oviduct and another when the egg was in the soft shell stage in the uterus. Birds P, Q, R, S were thus bled but once and the serum used for analysis. With the perfection of the operation it was found that the same bird could be used for both bleedings with no ill effect whatsoever to the bird. Therefore hen T was used for both an oviducal and a uterine bleeding, but in this case the oxalated plasma was employed for analysis. In all cases the blood was treated in a manner such as outlined above for the 26 hour experiment.

DISCUSSION OF RESULTS

The work done on this problem may be divided into four phases:

1. The study of the rise in protein phosphorus and calcium in the blood of a hen with the approach of the production period;
2. The study of the changes in the calcium and inorganic phosphorus content of the blood of the laying hen during the approximately 26 hours of a single egg cycle using non-laying hens for controls;

3. The study of the ultrafilterability of the inorganic phosphorus of the laying hen's blood at two different stages of the egg cycle; and
4. The study of the calcium and phosphorus content of the leg (ischiatic) artery and vein at two stages of the egg cycle.

When the first phase of this work was started the ten pullets used had not yet commenced to lay, but #1422 and #1427 were very close to the laying stage as evidenced by the high calcium level for these hens in Table 1. The fact that these two hens each laid their first egg the following week served to confirm the blood analysis. At the final bleeding all but two of the pullets had matured and were laying. From examination of Table 1 and of the several representative graphs included, it can be seen that there is a very close parallel between the rise in blood calcium and the appearance and rise of the protein phosphorus in the blood. Several workers (9, 10, 11) have demonstrated that the calcium rises greatly with the approach of productivity. Roepke (33) has shown that the protein phosphorus occurs in appreciable quantities only in laying hens.

Both these facts were confirmed here because it was found that in every case the appearance of a rise in the calcium and the finding of significant amounts of protein

Fig. 1. Rise in total calcium and protein phosphorus in non-production and production cycle in hen.

a. Hen No. 1424
b. Hen No. 1425
c. Hen No. 1426
d. Hen No. 1428

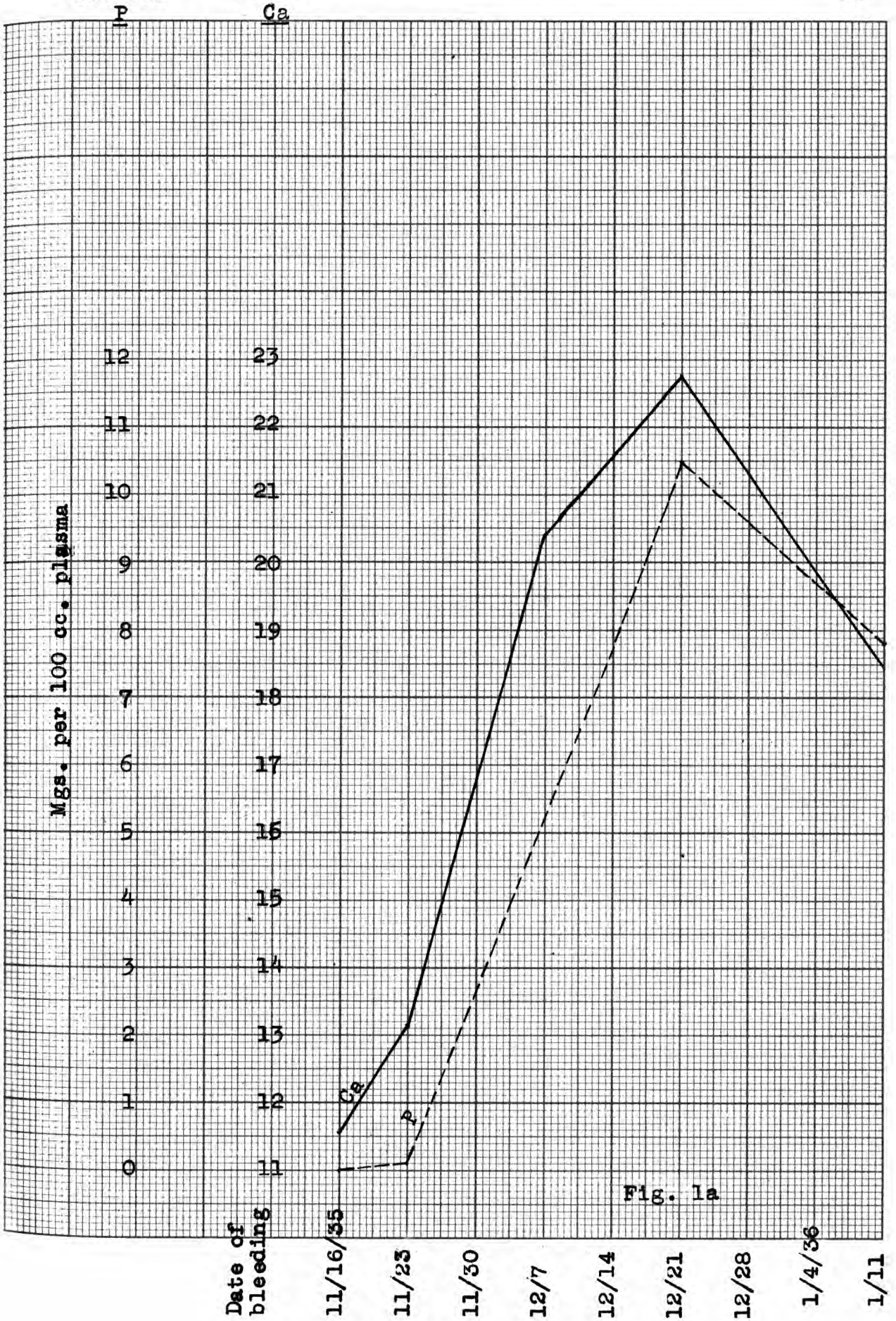
Protein
P
Total
Ca
Mgs. per 100 cc. plasma
12
11
10
9
8
7
6
5
4
3
2
1
0
23
22
21
20
19
18
17
16
15
14
13
12
11
Ca
P
Date of bleeding
11/16/35
11/23
11/30
12/7
12/14
12/21
12/28
1/4/36
1/11

Fig. 1a

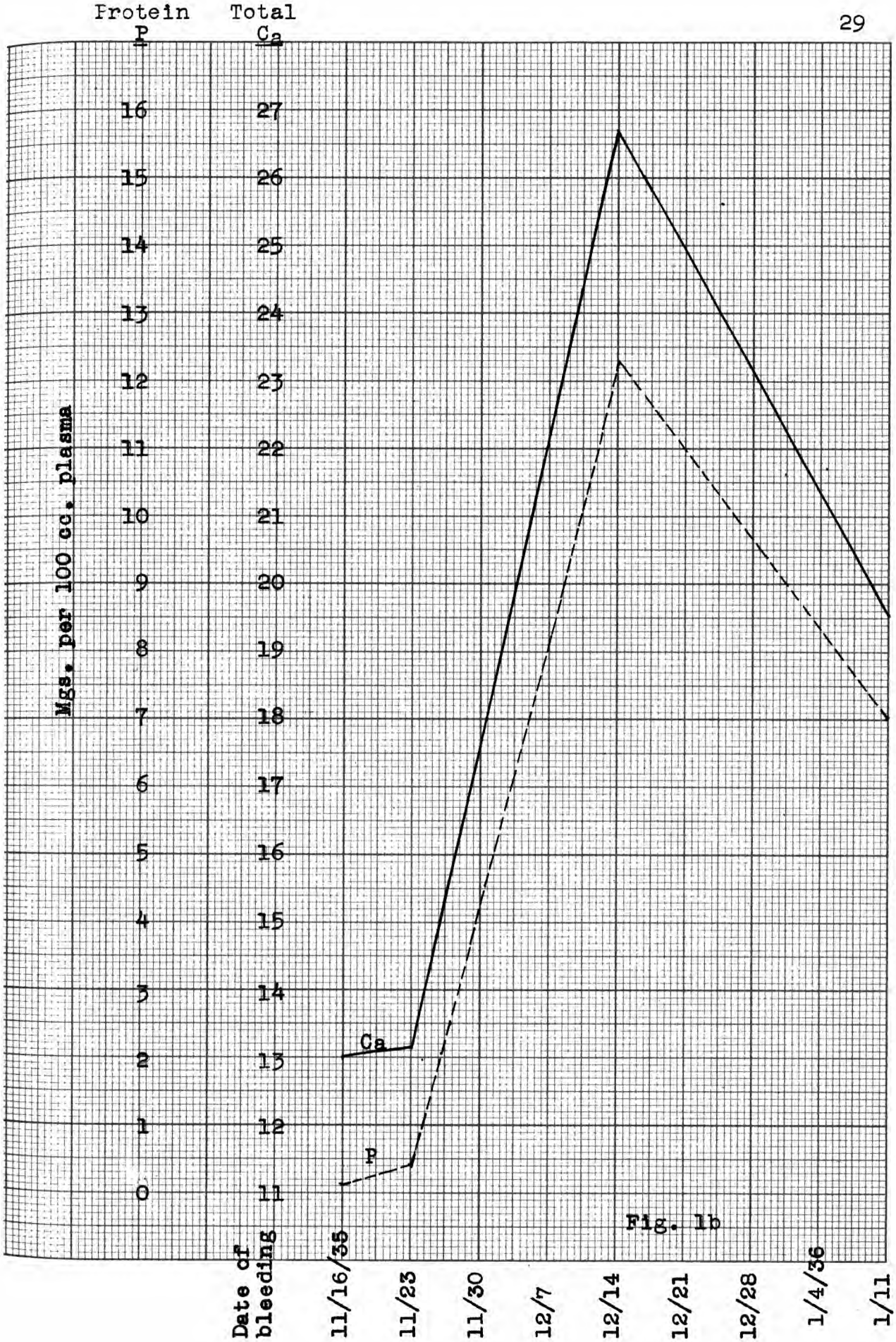
Protein P
Total Ca
Mgs. per 100 cc. plasma
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
27
26
25
24
23
22
21
20
19
18
17
16
15
14
13
12
11
Ca
P
Date of bleeding
11/16/35
11/23
11/30
12/7
12/14
12/21
12/28
1/4/36
1/11

Fig. 1b

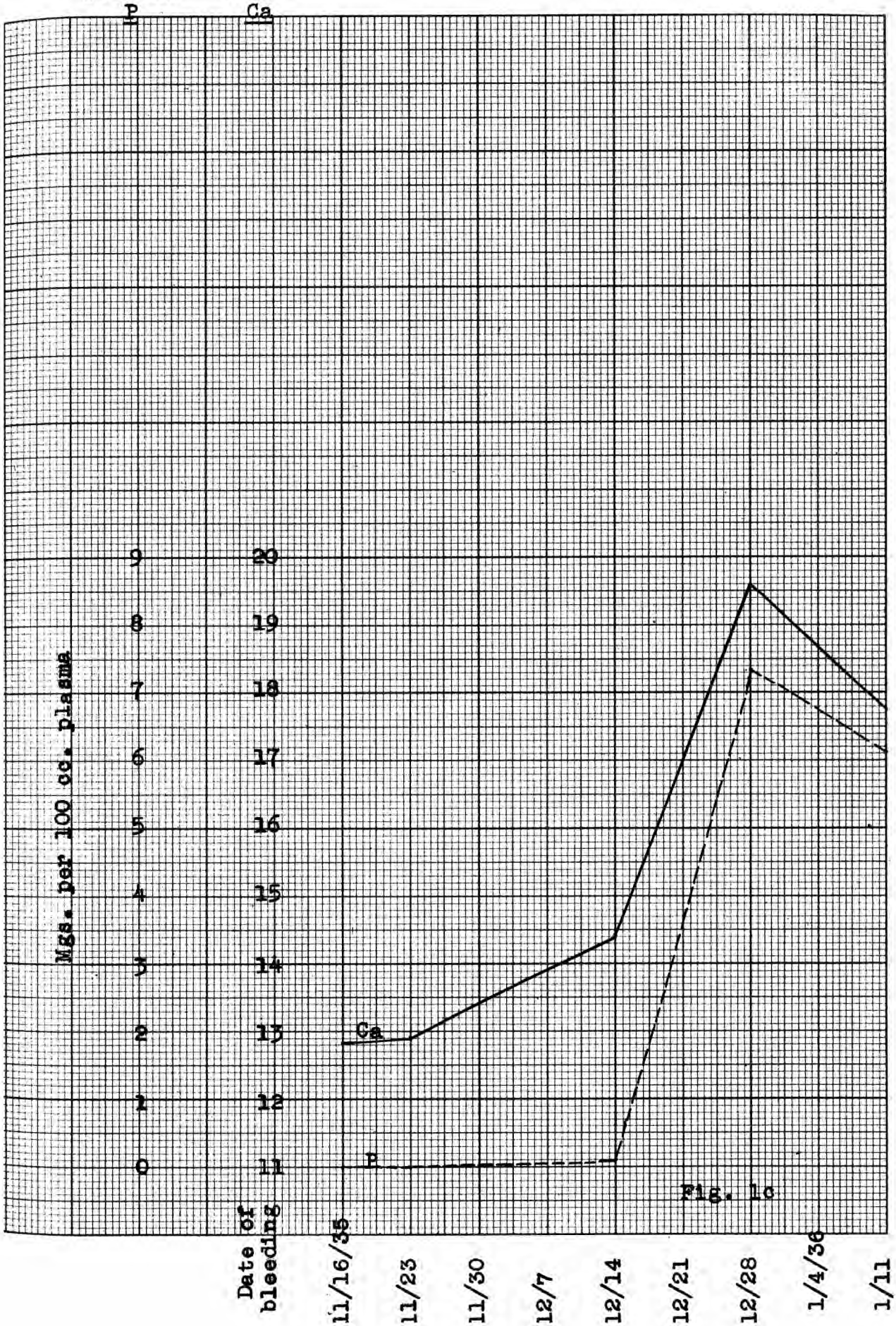
Protein P
Total Ca
Mgs. per 100 cc. plasma
9
8
7
6
5
4
3
2
1
0
20
19
18
17
16
15
14
13
12
11
Ca
P
Date of bleeding
11/16/35
11/23
11/30
12/7
12/14
12/21
12/28
1/4/36
1/11

Fig. 1c

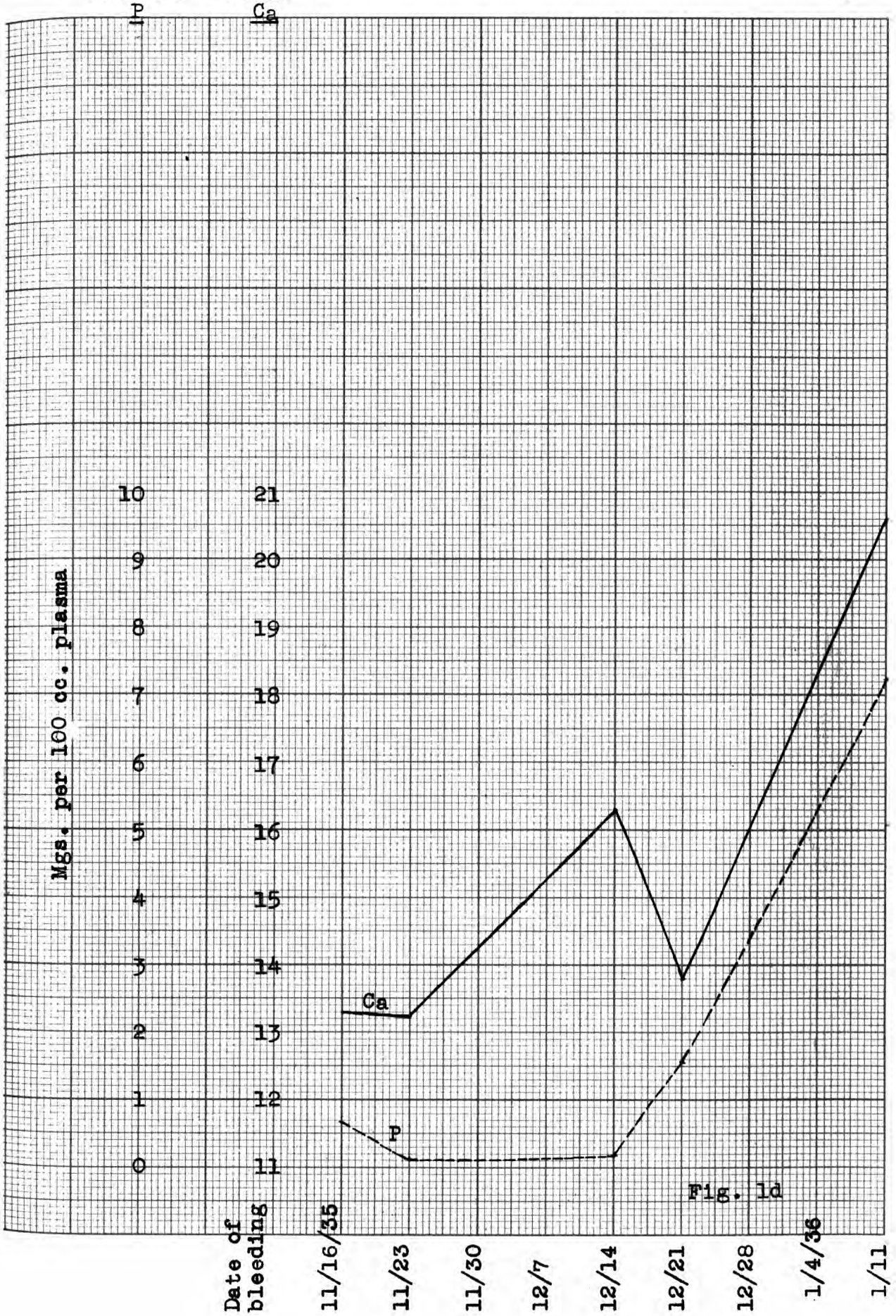
Protein P
Total Ca
Mgs. per 100 cc. plasma
10
9
8
7
6
5
4
3
2
1
0
21
20
19
18
17
16
15
14
13
12
11
Ca
P
Date of bleeding
11/16/35
11/23
11/30
12/7
12/14
12/21
12/28
1/4/36
1/11

Fig. 1d

Table 2

Ratio of Protein Phosphorus to Excess Calcium in Plasma.

Date	Hen No.	Protein Phosphorus	Total Calcium	Ratio P:Ca
11/16/35	1422	6.94	10.29	0.673
	1427	5.41	8.05	0.673
	1428	0.66	1.29	0.512
11/23/35	1423	0.65	2.23	.292
	1425	0.40	1.13	0.374
12/14/35	1420	7.27	11.26	0.646
	1421	1.49	4.04	0.369
	1423	6.13	11.00	0.557
	1425	12.22	14.66	0.835
12/21/35	1422	9.75	8.84	1.103
	1424	10.46	10.72	0.976
	1428	1.57	1.75	0.900
12/28/35	1420	12.17	13.96	0.872
	1426	7.33	7.60	0.965
1/11/36	1422	10.31	10.22	1.009
	1423	6.43	6.92	0.929
	1424	7.84	6.44	1.217
	1425	7.05	7.52	0.937
	1426	6.11	5.58	1.057
	1428	7.27	8.52	0.853

phosphorus was followed shortly by the beginning of production. This work demonstrated further that the rise in calcium and in protein phosphorus is simultaneous and that one closely parallels the other. This is brought out very clearly in the graphs. However, an attempt to establish a definite ratio between the excess calcium and the protein phosphorus was unsuccessful. In Table 2 the excess calcium was taken as that calcium over 12 mg. per 100 cc., the latter figure being a close average of the normal calcium content of the blood of non-laying hens. An examination of this table shows a wide range of ratios.

These results reveal, then, a close parallelism between the rise in calcium and in protein phosphorus in the laying hen. This suggests one of two possibilities--either the phospho-protein that makes its appearance in the hen's blood at the time of production is closely associated with the rise in calcium and acts as a carrier for the calcium, or else the two are associated with separate phases of egg production and their simultaneous rise is then a coincidence only. Which of these two possibilities is the correct one can only be ascertained by further research.

In studying the calcium and inorganic phosphorus content of the blood of the laying hen during the single egg cycle, using non-laying hens as controls for any possible

Table 3

Total Calcium Content of Serum during Twenty-Six Hour Period
(Mg. per 100 cc.).

Laying Hens	1	2	3	4	5	6	7	Per cent Difference between Low and High
A	21.1	19.9	19.2		19.5	18.6		13.5
B	24.2	23.0	21.9		22.2	22.6		10.5
C	21.2	20.2	19.9		18.6	18.2		16.5
D	32.4			32.4	32.4		29.6	9.5
E	31.0			31.2	30.5		28.4	9.9
F	19.9			21.1			18.2	13.8
G	23.0			24.1			23.4	4.8
Average								11.2
Non-Laying Hens								
V	16.2	15.2	16.2		16.3	15.7		7.2

Schedule of Bleedings:

1. Immediately after laying -- about 10:00 a.m.
2. 6-hour stage -- about 4:00 p.m.
3. 11½-hour stage (beginning of soft shell) -- about 9:30 p.m.
4. 13-hour stage -- about 11:00 p.m.
5. 17-hour stage -- about 3:00 a.m.
6. 21-hour stage -- about 7:00 a.m.
7. 26-hour stage -- about 12:00 noon.

diurnal factor, very interesting results were obtained. As seen from Table 3, the calcium level in the blood of both the laying and non-laying hens is quite constant, varying only within a few milligrams at the most. In computing the per cent difference between the high and low value an average of 11.2 per cent is obtained for laying hens while the one non-laying hen showed a difference of 7.2 per cent. Further, the change does not seem to be in any way connected with the stage in the egg cycle. These results agree with the findings of Deobald and his co-workers (7).

However, in studying the inorganic phosphorus variation a new and hitherto unreported fact (so far as any literature found on the subject is concerned) was discovered. Whereas the inorganic phosphorus level in the blood of the non-laying hen does not differ appreciably at different hours of the day and night, the inorganic phosphorus content of the blood of the laying hen increases greatly during those hours of the night at which the egg-shell is being laid down (Table 4). Thus, while the non-laying hens showed an average difference of only 13.6 per cent between the low and high phosphorus levels, the laying hens showed an average difference of 91 per cent.

Values were obtained for the phosphorus content which far exceeded previous reports on the inorganic phosphorus

Table 4

Inorganic Phosphorus Level during Twenty-Six Hour Period
(Mg. per 100 cc.).

Laying Hens	1	2	3	4	5	6	7	Per cent Difference between Low and High
A	3.56	4.32	8.54		9.14	8.00		157.
B	3.90	4.88	7.81		7.67	4.47		100.
C	4.42	4.30	5.52		4.73	4.62		28.
D	5.88			8.55	7.66		6.63	44.
E	4.23			8.74	9.70		4.52	129.
F	3.40			7.51			3.33	126.
G	4.30			6.32			4.42	47.
H	4.04			7.21			5.06	76.
I	5.24			9.04			4.12	120.
J	3.46			6.15			3.88	78.
Average								91.
Non-Laying Hens								
V	3.25	3.49	3.52		3.56	3.40		9.5
W	4.82			5.60			5.33	16.2
X	2.57			2.77			2.89	12.5
Y	4.21			4.50			4.60	9.3
Z	3.78			4.57			4.26	20.4
Average								13.6

Schedule of Bleedings same as for Table 3.

content of the blood of the laying hen. The following figures have been reported by various workers: 4-6 mg. by Russell, Massengale, and Howard (35); 2.50-5.15 mg. by Hart, Halpin, and Steenbock (36); 3.55-5.10 mg. by Hughes and Titus (37). Dyer and Roe (38), working on the blood constituents of normal chickens, found the inorganic phosphorus ran from 2.7 to 5.9 mg. with an average of 4.6. Yet, despite these reported figures, the author found that during shell deposition the phosphorus rose as high as 9.70 mg. for one hen and frequently went to 8 and 9 mg. in others. The reason these high values were not detected by previous workers is that they occur during the night, at times when such work would not usually be carried out.

The results obtained are very interesting when compared with the findings of Common (4). In this paper he reports a great increase in the phosphorus content of the excreta of hens during egg laying, and that the time of the excess corresponds with the period of shell formation. He suggests that the source of the excreted phosphorus is the bone, $Ca_3(PO_4)_2$, which is being broken down for calcium for the shell.

It is of interest and worthy of note that working on blood, the author reached the same conclusion as Common did working on excreta. Before coming across Common's work the

Table 5

Ultrafilterability of Inorganic Phosphorus in Serum at High (Soft Shell) and Low (Oviducal) Phosphorus Levels.

Hen	Egg Stage	Mg. Ca per 100 cc. Serum	Mg. Ca per 100 cc. Ultrafiltrate	% Total Ca That Is Ultrafilterable	% Increase of Ca in Ultrafiltrate	% of Excess Ca That Is Ultrafilterable
L	Oviducal	3.84	2.55	66.	23.5	34.1
	Soft Shell	5.60	3.15	56.		
M	Oviducal	6.06	3.15	52.	34.3	43.2
	Soft Shell	8.56	4.23	49.		
N	Oviducal	3.65	2.35	64.	30.6	40.3
	Soft Shell	5.44	3.07	56.		
Average	Oviducal			61.	29.5	39.2
	Soft Shell			54.		

author, upon finding the great rise in inorganic phosphorus at the time of shell deposition, suggested that the source was the bone reserve upon which the hen was drawing for calcium, and that since there was this rise in blood phosphorus there would probably be an increase in phosphorus excretion at this time. It was gratifying, therefore, to find that Common's work corroborated these theories.

Upon finding this great rise in inorganic phosphorus the question naturally arose as to whether it was filterable or non-filterable in nature. Owing to the small size of the ultrafilter, the length of time required for each sample, and the small amount of ultrafiltrate collected, only three hens were used and only the phosphorus was determined, though the determination of the filterability of the calcium would have been of great interest.

Looking at the results in Table 5 we find that at the low phosphorus stage an average of 61 per cent of the phosphorus is ultrafilterable. This figure is high compared with the 36.5 per cent reported by Roepke (33) and the 54 per cent reported by Laskowski (39), but falls below the 64.5 per cent reported by Benjamin and Hess (13), and lies reasonably well between the latter two figures. The per cent ultrafilterability of the inorganic phosphorus at the high stage, however, is found to be only 54 per cent. This at

first seems anomalous since it would be expected that if the high inorganic phosphorus level is due to the liberation of phosphate ion from the $Ca_3(PO_4)_2$ of the bone, the phosphorus should be in a filterable (ionic) form.

Upon considering other factors and the work of other men, though, these results are perfectly reasonable and in accordance with the work of others. In the first place it would be unreasonable to believe that such a large excess of a diffusible form of phosphorus would be allowed to accumulate by the kidneys. Such a high concentration of phosphate ion would be dangerous because it would undoubtedly repress the calcium ion concentration of the blood to a pathological level. One would expect, though, that with the rapid liberation of phosphate ions there would be some increase in the filterable phosphorus. This expectation is carried out by the fact that there is an average rise of 29.5 per cent in filterable phosphorus in going from the low stage to the high stage.

Secondly, this low ultrafilterability of the excess calcium is to be expected when considered in the light of Smith's work (21). The latter found that in the presence of excess inorganic phosphorus in serum, excess calcium (which is present in the blood of the laying hen) combines solely with the phosphorus until the phosphorus has reached a normal

Table 6

Total Calcium and Inorganic Phosphorus Level in Ischiatic Artery and Vein at Two Stages in Egg Cycle.

		Oviducal Stage						Soft-Shell Stage					
		Calcium			Phosphorus			Calcium			Phosphorus		
Hen		Content	Mg. Difference	% Difference	Content	Mg. Difference	% Difference	Content	Mg. Difference	% Difference	Content	Mg. Difference	% Difference
P	Artery	27.20	1.98	7.20	4.79	0.35	7.50						
	Vein	25.32			4.44								
Q	Artery							16.40	0.84	5.1	6.75	0.39	5.6
	Vein							17.24			7.14		
R	Artery	19.70	0.84	4.40	3.04	0.07	2.30						
	Vein	18.86			2.97								
S	Artery							27.98	3.02	10.8	8.65	0.49	5.5
	Vein							31.00			9.14		
T	Artery				2.25	0.07	3.20				4.32	0.56	12.2
	Vein				2.18						4.88		
Average			1.41	5.80		0.12	4.50		1.93	8.0		0.48	7.8

level. It is to be noted that the final ultrafilterable phosphorus level at the high stage is 54 per cent which, according to the report of Laskowski (39), is a normal level. This 54 per cent does not differ greatly from the 61 per cent ultrafilterability of the phosphorus at the low stage. The results obtained here, then, seem to be an "in vivo" corroboration of Smith's "in vitro" work.

The final phase of this work was carried out to try to demonstrate the source of the calcium for the egg shell. Deobald, et al (7) had reported a 10 per cent increase in the ash content of the bones of the laying hens, suggesting this medium as a means of calcium storage for future use. Kyes and Potter (6) narrowed the place of storage down, in the case of pigeons, to the marrow space of the long bones of the leg--specifically the femur. It seemed probable then that if the hen stored her calcium here she also drew upon it when the need arose. Therefore there should be a demonstrable difference between the phosphorus and calcium content of the vein and artery supplying the leg (including the bones).

In Table 6 are given the results of analysis of the blood of the ischiatic artery and ischiatic vein of laying hens at a shell-depositing and a non-shell-depositing stage. The results show that at the time of shell deposition there

was an excess of calcium and phosphorus in the vein over the artery, while at the time when no shell was being deposited there was an excess in the artery over the vein. This would indicate that when there is no demand for calcium it is carried to the bones and stored as calcium phosphate, while when there is a great demand for calcium at the time of shell deposition it is taken from the bones as the phosphate and carried to the uterus.

The differences in the calcium levels are fairly large, averaging 1.41 mg. at the oviducal stage and 1.93 mg. at the soft-shell stage. The differences in the phosphorus are much smaller, being only 0.12 mg. in the oviducal stage and 0.48 mg. in the soft shell stage. From a percentage viewpoint, though, there is not so much difference between the calcium and the phosphorus, there being a 5.8 per cent difference in calcium and a 4.5 per cent difference in phosphorus at the oviducal stage, and an 8 per cent difference in calcium and a 7.8 per cent difference in phosphorus at the soft-shell stage. However, the most significant feature of these results is not the <u>amount</u> of the difference but rather that the differences were consistently in the same direction. It is to be noted that the ratio of Ca:P is not that of $Ca_3(PO_4)_2$. The explanation for this will necessitate further research.

The results obtained from this work may now be regarded with the idea of pointing out a possible mechanism for metabolism of calcium by the laying hen for egg shell formation. A probable explanation for this phenomenon would seem to be as follows. As productivity approaches there is a rise in the protein content of the blood due to the appearance of a phospho-protein. It is the belief of the author that this protein is related to yolk formation rather than to calcium metabolism, since Roepke and Bushnell (31) showed it to be identical with the vitellin of the egg yolk. However, the presence of the protein in the blood acts as a mechanical factor in the increase of calcium in the blood owing to the adsorption of the calcium on the protein. This, according to Benjamin and Hess (13), accounts for about 50 per cent of the excess calcium. The remainder of the excess calcium is to be regarded as tied up in a non-filterable, adsorbable calcium-phosphorus complex (11, 13, 15, 21). At any rate, the rise in calcium is in the non-diffusible form (12), since the hen could not tolerate a great rise in ionized calcium.

The purpose of the calcium rise would be to supply an immediate source for the start of shell deposition. This is substantiated by the fact that Heller, Paul, and Thompson (11) concluded from their work that it was the non-filterable, adsorbable calcium of the blood that is concerned with

shell formation. However, even with this large excess of calcium in the blood there is but a matter of milligrams of calcium in the whole blood stream--just a minute fraction of the amount necessary to make a single egg-shell. It is evident, then, that the hen must have available a ready source of calcium at the time of shell deposition. This source may lie in one of two places--the feed in the intestinal tract and the $Ca_3(PO_4)_2$ depots of the body (the bones). Buckner, Martin, and Hull (23) showed that there was an active absorption of calcium from the intestine of the laying hen, but this absorption is just as rapid when the egg is in the isthmus as when it is in the uterus and the shell is being deposited. Common (5) stated that if the hen ingested "sufficient" calcium it would not have to draw on its body reserves of calcium. Undoubtedly some of the calcium used in egg-shell formation may come directly from the intestine at the time of shell deposition.

But, that this is not the principal source is indicated by the fact that the laying bird goes to the trouble to store up a supply of calcium in its bones. More direct evidence is found in Common's work (4, 5, 24), where it is demonstrated that on normal diets egg-shell formation is accompanied by an excess excretion of phosphate in the droppings, this excess phosphate coming presumably from bone that is being

broken down to liberate calcium for the shell. That the hen is capable of breaking down this bone as needed is shown by the large increase in serum phosphatase in the laying hen (8).

The work presented here on the comparison of the calcium and phosphorus in the ischiatic artery and ischiatic vein during shell-deposition and during non-shell-deposition furthers this view by indicating that the hen stores calcium in her leg bones when not using it for shell formation and later withdraws this calcium as needed for shell deposition.

It would appear probable, then, that at the start of shell deposition the high excess of calcium in the blood stream is drawn upon. This lowers the calcium level momentarily and starts the mechanism for the calcium removal from the bone reserve. As the calcium for the shell is removed, phosphate is set free and appears as an increase in the inorganic phosphorus content of the blood (this paper) and an increase in the phosphate content of the excreta (4, 5, 24). In the period of rest the hen builds back her reserve of calcium in the bones.

Again it is of interest to note that a very similar theory was evolved by Common (5) through analysis of excreta. That both blood analysis and excreta analysis lead to the same theory would tend to lend some credence to it.

SUMMARY

This research has demonstrated: (1) a parallelism between the appearance of a phospho-protein and a rise in the total calcium in the blood of the hen as she reaches maturity and comes into production; (2) a relative constancy of the calcium in the blood of laying and non-laying hens during the 24 hour day and during the single egg cycle of the laying hen, confirming the findings of Deobald and his co-workers; (3) a marked rise in the inorganic phosphorus content of the blood of laying hens during egg-shell formation without a similar rise in the blood of non-laying hens at a similar hour of the day; (4) the per cent ultrafilterability of the phosphorus at the high stage is about 7 per cent less than that at the low stage; and (5) the calcium and phosphorus are higher in the ischiatic artery than in the ischiatic vein when the egg is in the oviduct, but the reverse is true when the egg is in the uterus and the shell is being formed.

CONCLUSIONS

Analysis of the results obtained lead to the following conclusions:

1. Calcium and phosphorus are stored in the long bones

of the hen as calcium phosphate during that period of the egg cycle when the shell is not being formed and there is an excess of calcium intake over calcium output.

2. During egg-shell formation, when there is a great demand for calcium, the hen draws upon the reserve previously stored in the bones.

3. The calcium content of the blood is constant, even during egg-shell formation, showing that the hen has available a ready store of calcium from which she can draw calcium as rapidly as it is being deposited in the shell.

4. The phosphorus rise is due to the liberation of the phosphorus from the calcium phosphate when the calcium is removed to be laid down as calcium carbonate in the shell.

5. A theory as to the possible mechanism of egg-shell formation is presented.

ACKNOWLEDGMENTS

The author wishes to express his thanks here to those fellow students and members of the faculty who advised and assisted him in the course of his research. Especially is he indebted to Prof. J. S. Hughes, who suggested the course of this work, and to Prof. H. M. Scott, who cooperated on the poultry phase of the problem and who, by his demonstration on the anatomy of the leg of the fowl, made possible the perfection of the surgical operation for the drawing of blood from the ischiatic vessels.

To Miss Helen Higbee he extends his sincere thanks for her assistance in transcribing notes and in typing the original draft of this manuscript.

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