THE CALCIUM PARTITION IN THE BLOOD SERUM OF CHICKENS

by

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INTRODUCTION AND HISTORICAL

June 30, 1808, Sir Humphrey Davy (1) in a lecture before the English Royal Society stated that he had succeeded in decomposing the alkaline earths. He stated, "I shall venture to denominate the metals from the alkaline earths barium, strontium, calcium....."

Since then calcium and its abundant compounds have been the subject of much exhaustive research, its physical properties being first accurately determined by Erdmann and Marchand (2) by the ignition of calcspar.

Early analytical work in the field of physiological chemistry soon revealed the presence of this important element within the living body in several forms. It was found to be the major inorganic constituent of bone as tri calcium phosphate and calcium carbonate; as calcium fluoride it has been shown to exist in the teeth; its salts are known to exist in the tissues and it was soon shown to be present in solution in the plasma. In each of these it has been assigned a definite role though many of these physiological actions are still the subject of research. To Arthus and Pages (3) is assigned the proof that the calcium salts are essential to the process of normal blood

coagulation. Clark (3), Mines and others have shown that calcium salts play a most important role in connection with the irritability of muscle and nerve; increasing amounts of calcium increase the contractions of the heart muscle, bringing on so-called calcium riger. Numerous investigators have shown that this element is controlled by at least two governors in the body, being subject to hormone and vitamin regulation. the recent work of Kozelka, Hart and Bohstedt (4) forcefully showing the interrelationship of the hormone of the parathyroids and vitamin D in controlling calcium concentration in the blood.

In 1911 Rona and Takahashi (5) published an article in the Biochemical Zeitschrift establishing the now well recognized fact that calcium in the plasma does not all exist in the form of simple solution. By dialyzing serum in which all inorganic salts except calcium were fully compensated for in the dialyzing medium outside the membrane, while calcium was present in varying amounts, the authors showed that on the average only 65% of the calcium in serum was capable of diffusing through a membrane. In rapid succession Cushny (6), Richter-Quittner (7), Kirk and King (8) and numerous others have studied this phenomenon, attempting to explain it and to associate it with pathological conditions, such as rickets, epilepsy, etc.

Past research work on the calcium content of the blood in the aves has established the interesting fact that the calcium concentration varies over a large range in the serum of the female, depending upon the activity of the ovaries. The total calcium of a productive hen runs two to two and a half times as high as that in the non-productive bird.

Subsequently it is the purpose of this thesis to establish the amount of diffusible calcium in fowl serum at the varying concentrations of total calcium, hence to determine whether or not the diffusible or non-diffusible portion fluctuates and, if possible, deduce the cause.

REVIEW OF LITERATURE

Several methods have been used by investigators to determine whether the salts present in the blood are there in the form of simple solution or whether they are bound by the proteins in an "ion-protein" complex. Conspicuous among the several methods is the compensatory dialysis used by many experimentors. The electrochemical study of electrolytes in the blood has also been used, while ultrafiltration of serum with pressure is a third practical method.

Rona and Takahashi (5) in 1911 published an article previously referred to, in which they described their method of subjecting serum to compensatory dialysis. In this way

they first showed calcium to be present in two forms, since only 65% of the total calcium was capable of passing through a membrane. Other investigators have used this and similar methods obtaining results varying somewhat in the per cent of diffusible calcium found.

In 1919 Cushny (6) published an article describing a method of determining the colloid constituents of the blood serum. By their method blood serum was filtered through a carefully prepared collodion membrane at a pressure of 150 mm. of mercury. In this way a clear protein-free filtrate was obtained. Of the results of this filtration he writes, "When serum is filtered through a collodion membrane which retains the colloids, most of the other constituents, such as salts, sugars, urea, occur in the filtrate in the same proportions as in the original serum. The only exceptions to this rule are calcium and possibly magnesium which pass through the filter in a lower concentration than exists in serum."

This general method of determining the diffusible salts, or more appropriately, the filterable salts, has probably been the most widely used. Updegraff, Greenberg and Clark (9) describe a method of making the collodion sacs with two dippings of the collodion solution. An ingenious apparatus using a flask to maintain the proper CO_2

tension during filtration is described by Greenberg and Gunther (10). Shih-Hao Liu (11), Meysenbug, Pappenheimer, Zucker and Murray (12), along with many other investigators have used this type of ultrafiltration to determine the mineral partition in the blood. The diffusible calcium reported by these investigators ranges from 51 per cent (11) to around 70 per cent (12).

In 1932 Nicholas (13) published an article describing a method for filtering blood serum through a filter of du Pont number three hundred cellophane under high pressure. By this method a clear protein-free filtrate is obtained that gives uniform results. It is this type of filtration that was used to collect data for this thesis.

Neuhausen and Marshall (14) in an electrochemical study of the condition of several electrolytes in the blood by using different electrodes came to the conclusions "...that the sodium and chloride are present as in an aqueous solution of sodium chloride (and sodium bicarbonate) of the same concentration, while only about 10 per cent of the total calcium is present in ionic form." They did, however, express difficulty in determining the calcium ion due to the presence of other alkali cations besides the one for which the amalgam is a reversible electrode. In a later publication Neuhausen and Pincus (15) state that owing to

the number of ionic constituents present in the serum, only a few of the ionic species can be determined electrometrically and they suggest: "The inorganic constituents of the serum may be present in three forms: as ions, as undissociated molecules in equilibrium with the ions, and as non-ionizable compounds with some of the organic constituents." By ultrafiltration they decided the calcium in pig serum seemed to be from 50 to 80 per cent diffusible.

Each of the discussed methods of procedure appears to have its shortcomings. To dialize the serum is open to attack because of the difficulty in compensating for all the organic constituents found in serum. Variable results in the case of inorganic constituents are also obtained due to the existence of a Donnan membrane equilibrium on the sides of the collodion membrane (15). To obtain uniform collodion membranes has been another difficulty met by these investigators.

This same objection holds for the use of this membrane to obtain ultrafiltrates at low pressures. With such lack of uniformity in permeability of the membranes varying, even low pressures, seemed likely to give some variance in results. And since the collodion sacs held some moisture the first few drops have to be wiped off them to insure an undiluted ultrafiltrate.

The electrochemical method is as stated by users (14) at best only a measure of the actually ionized salts and does not necessarily give a true picture of the diffusible and non-diffusible constituents present in the serum.

The use of high pressures running into tens of atmospheres to obtain ultrafiltrates through Bechold filters has been criticised by Burian (16) and others on the basis that labile compounds between the proteins and inorganic ions may have been decomposed by the excessive pressures

According to Greenberg and Gunther (10), "This point of view is not well grounded. Since from the thermodynamic standpoint the only influence that pressure can have on equilibrium in aqueous solution is by changes produced in the partial molal volumes of the constituents, in the light of the small compressibility of such solutions it is obvious that pressures of even several atmospheres can have little effect on the values of diffusible calcium. Where differences have been claimed as in the low results obtained by Richter-Quittner with a low filtration pressure, the results are probably due to imperfect filtering membranes."

Nicholas (13) has shown that pressures varying from 50 to 200 pounds per square inch had no effect on calcium distribution. By using cellophane filters cut from several different parts of a roll, he showed the uniformity of the

filter, and by using a standard solution of calcium chloride showed that no calcium was adsorbed by the filters.

Therefore, the use of an ultrafilter such as was used by Nicholas and as was used in this research has the distinct advantages, in using du Pont 300 cellophane as a filter, of being more conveniently handled, saving the preparation of collodion sacs, being more uniformly permeable, and the filter is dry, leaving no question as to the concentration of the filtrate. Results obtained by this method seemed to fall within the normal range accepted by experimentors, and the values obtained, about 64% diffusible calcium, according to Nicholas (13) are more constant.

The nature and activity of the different forms of calcium in the blood serum have been the subjects of much exhaustive research. The non-diffusible form of calcium has been more and more referred to in the literature as the protein-bound calcium, though actual proof of the existence of such an arrangement is still lacking and some authors disparage the use of such a term.

The term protein-bound calcium finds favor in the literature (17) because it is known that proteins decrease the diffusibility and increase the solubility of calcium, and because of the relation of calcium and protein in certain pathological conditions.

The other form of calcium is usually referred to as ionic calcium, though as we have seen all the diffusible calcium is said not to exist in the ionized state; hence we really have three forms.

According to Marrack and Thacker (18), "Calcium forms an un-ionized compound with proteins.....The excess of calcium in protein solution is independent of the bicarbonate and phosphate concentration."

At 37 degrees and pH 7.3 to 7.55 they give the following formula:

$$\frac{Ca_P - Ca_S}{Ca_S\ P} = 0.11$$

in which Ca_P is the protein-bound calcium and Ca_S is the calcium in solution. They also stated that, "The excess of calcium in the protein solutions is far greater than the Donnan equilibrium will account for.....That is the difference of concentration produced by the Donnan effect is almost negligible."

Greenberg and Gunther (10) also believe that the non-diffusible calcium is protein-bound. Using the data of Loeb and Nichols corrected for serum protein volume in the form of the Langmuir adsorption isotherm they obtain

$$\frac{(Ca)_P}{P} = \frac{(\overset{++}{Ca})}{52.6 + 29.4\ (\overset{++}{Ca})}$$

From this they conclude that, "...in blood serum the limiting amount of calcium that can be bound by the protein, i.e., the non-diffusible calcium, even if the calcium concentration is infinitely increased, is not of much greater magnitude than the non-diffusible calcium already present. ...the limiting value of protein-bound calcium when the ionic calcium approaches infinity is of the same order as is present in normal blood serum."

In support of this view Smith and Sternberger (19) report on a series of intravenously injected calcium solutions into dogs, in which they observed no increase in per cent of total calcium being diffusible. They write, "When the serum calcium content of normal dogs is increased by intravenous injection of wholly diffusible calcium salts, the relationship between diffusible and non-diffusible calcium assumes approximately normal proportions, explainable by an equilibrium between calcium and serum protein. This indicates that the normal serum protein is not saturated with calcium but under the above conditions is capable of combining with more than the normal amount." They reproduced the above finding in vitro.

This view is further substantiated by the work of Morgulis and Perley (20) in dialyzing blood serum against equal volumes of cerebrospinal fluid.

According to Greenberg and Greenburg (21) there are many writers, including Greenwald, Sendroy and Hastings, Bernhard and Beaver, and Brull, who support a hypothesis that a considerable fraction of the diffusible calcium of the blood is present in a complex, negatively charged ion, in combination with some unknown organic substance--a citrate-like calcium compound. The authors conclude, however, that there is no strongly positive evidence for the existence of such a compound. Neuhausen and Marshall (14) whose work has been earlier referred to, support the theory of an undissociated calcium molecule.

From the above contributions to the literature then it could be concluded that the protein-bound calcium and the diffusible calcium are in equilibrium with each other and that an increase in the total calcium means an increase in both fractions.

It might be expected that changes in the CO_2-combining power and capacity of the blood serum, by affecting a change in the pH, and hence the solubility product, of the inorganic salts in the serum, would vary the amount of diffusible calcium. Though theoretically such an argument is sound, the results of practically all the investigators show that the CO_2- tension at filtration makes no difference as to the results that might be expected. Such observations

have been reported by Meysenbug and McCann (22). Marrack and Thacker (18) have shown that the calcium ion concentration of body fluid is not regulated by the solubility of calcium carbonate and calcium phosphate, while Greenberg and Gunther (10) state that changing the CO_2-tension between the range pH seven to eight has no influence on the distribution of calcium between diffusible and non diffusible in the time allowed for ultrafiltration.

It has been a subject of much research to determine which calcium fraction is most pathologically active and whether one or both diffusible and non-diffusible fractions change in pathological conditions.

According to Shih-Hao Liu (11) in some cases diffusible calcium fluctuates, decreasing more than the non-diffusible in tetany. However, most articles seem to conclude that in general any change in the total calcium cannot be credited to either fraction. Meysenbug and McCann (22) state that Howland and Warriott (1917) found a slight diminution of the serum calcium in rickets; the ratio of the diffusible to non-diffusible was in the normal range. Likewise, they, themselves, found in cases of experimental tetany in dogs a fluctuation in the total serum calcium, but a constancy of the ratio of non-diffusible to diffusible calcium. Marrack and Thacker (18) state that epilepsy is not caused

by a reduction of calcium ion concentration in body fluids.

In a more recent article Benjamin and Hess (17) consider three forms of calcium in the body, the protein-bound calcium, the calcium ion, and an adsorbable calcium-phosphorous complex present in both the diffusible and non-diffusible portions. They report that it is in the adsorbable fraction of the calcium where the diminution in concentration is observed in rickets regardless of the level of the total calcium and phosphorous.

Benjamin (23) also reports that, "The property of adsorbing the calcium-phosphorous complex was found to be limited to the part of the cartilage in which calcification normally occurs; namely, the region of the junction of epiphysis and metaphysis. Resting cartilage from the epiphyses of long bones as well as non-calcifying rib cartilage failed to adsorb the complex."

The nature and condition of calcium in the blood serum of the aves has been shown to be quite different from normal mammalian blood. Riddell and Rheinhart (24) published a report showing that there was a marked rise in blood calcium in pigeons at the time of egg production. Hughes, Titus and Smits (25) report that young chicks, capons, immature and mature cockerels, immature pullets and molting hens not in production all have plasma calcium

contents of 12 to 14 mg. per 100 cc., which is only slightly higher than is expected in mammalian plasma. They further report, however, that mature pullets and hens in production have calcium in their plasma ranging from 13 mg. to 36 mg. per 100 cc. of plasma. Buckner, Martin and Hull (26) report similar results. They state that the calcium is excreted as the carbonate in the egg shell, there being about 5.5 grams of calcium carbonate in the shell of a normal egg. There are no calcium or protein storage organs in the chicken, according to the above. They write, "The actual time of secretion of these 5.5 grams of calcium carbonate as egg shell is estimated at not more than sixteen hours. This calcium is delivered by the blood stream to the oviduct where it is secreted principally as the egg shell." The fluctuation of blood calcium in the hen over large ranges is confirmed by the results of Russell, Howard and Hess (27). They report a relationship between the blood calcium in the hen and the size of the egg in the ovary, an ovum greater than one centimeter in diameter being accompanied by a blood calcium level between 13.0 mg. to 26.7 mg. per 100 cc. of serum. Their data indicate, however, that the presence of large ova and high blood calcium does not always indicate active egg production. They conclude by writing, "Whether the development of ova

caused the blood calcium to rise or whether an increase in blood calcium stimulated ova formation will have to be investigated further, but it should be noted that high blood calcium was never found unless developed or developing ova were present."

Calcium content of normal mammalian blood is very constant, seldom varying more than a milligram from 10 mg. per 100 cc. of blood, except in pathological conditions, and then only a slight variance is accompanied by the most serious results. Hence, it appears peculiar that normal hens can have blood calciums, ranging from just a little higher than that which is found in mammalian blood, to two and a half to three times as great and still be perfectly normal fowls.

That the blood calcium of hens varies over a wide range while the calcium of other fowls is constant is well established. No references, however, were found as to the nature of the partition of this calcium in chicken serum, and as to whether the diffusible or non-diffusible calcium or both fluctuated with the total. Therefore, it remained to make a study of the calcium partition in chicken serum, to determine, if possible, how the hen could carry such a high calcium content in her blood, studying the relation of the blood proteins and calcium fractions to the fluctuating total serum calcium.

THE ULTRAFILTER

The high pressure ultrafilter used for the determination of diffusible serum calcium was of the same type as that described by Nicholas (13), with slight modifications. It is simply a diminutive form of the usual large size high pressure ultrafilter.

The two pictures in Plate I show the set up of the apparatus. The upper picture shows the filter completely assembled as it is when in use. The picture at the bottom gives a view of the upper and lower sections when separated; the filter pieces can be seen between the two.

It will be seen that the apparatus consists of an ordinary two inch gas union with reducers. The upper Y leads to the gauge, nitrogen tank, and furnishes the opening through which the sample is introduced. The ultrafiltrate is collected in a test tube suspended from the lower delivery pipe.

The space in the gas union, ordinarily occupied by a gasket, was milled to hold the filter pieces. Two pins project from opposite sides of the lower rim, pass through holes in the edges of the filtering pieces, and enter opposite holes in the rim above. This prevents any twisting or tearing of the cellophane membrane while in the process

Plate I

of tightening the union.

As shown in the picture the filter pieces consist of a solid silver, cast plate, drilled with numerous holes, a silver plated copper gauze of about 15 mesh, a piece of high grade ashless filter paper, a cellophane membrane, cut from du Pont 300 plain cellophane, and finally, a rubber gasket.

In the ultrafilter first used for this work, oxygen was used to furnish the pressure in place of nitrogen, the mesh gauze was unplated, and an iron plate was used in place of the solid silver one. Use of these parts gave rise to cloudy and discolored filtrates. The solutions obviously contained some iron hydroxide, and results from these filtrates were hard to check. In comparison with later work all the values obtained for diffusible calcium on such filtrates were several milligrams low. To centrifuge out the foreign material aided in making the determinations, but did not raise the value of the results obtained, which were obviously low.

The probability that some colloidal iron compounds were adsorbing some of the calcium in the filtrate is made to appear even more likely in a recent article by Benjamin and Hess (17) which describes an adsorbable calcium fraction in ultrafiltrates.

Since it was suspected that the iron hydroxide might be adsorbing some of the calcium, the tank of nitrogen was obtained to furnish the pressure; while the copper mesh and iron plate were silver plated. It was found difficult, however, to silver plate into the holes of the iron plate. To eliminate this trouble a solid silver plate was milled, and very constant and satisfactory results have accompanied its use.

To use the filter it is completely assembled as shown and a quantity of serum introduced into the filtering chamber. The air in the chamber is blown out, then the pressure is raised to about 150 pounds and held around there. The ultrafiltrate comes through water clear, and on several different occasions the filtrate was tested by the biuret test and it always proved to be protein-free.

EXPERIMENTAL PROCEDURE

Calcium determinations were made on the blood serum, the subject being bled from the heart with a syringe. Between twenty to thirty cc. of blood were taken, considerably weakening the chicken but seldom sacrificing it.

The blood was then allowed to stand fifteen to twenty minutes to insure complete clotting; the clot was broken occasionally by a stirring rod, though care was taken not

to lake the blood. It was observed that if sufficient time was not allowed for coagulation, the serum obtained might itself clot and ruin the sample. Greenberg and Gunther (10) report that once the blood clot is formed the time of contact of the serum with the clot has no effect on the total calcium or its distribution for periods of twenty-four hours or even longer. However, after coagulation the serum was usually separated at once by centrifuging, the material being syphoned off into a small test tube. The total calcium determinations were set up at once and the remaining serum put in the ice box for use on the slower diffusible calcium determinations.

Total calcium determinations were usually made by the Kramer and Tisdall (28) method, as modified by Tisdall (29), by Clark and Collip (30), and by Standford and Wheatley (31). By this method all the calcium present in the serum, no matter how it is bound, is precipitated according to the equation:

$$CaX_2 + (NH_4)_2C_2O_4 = CaC_2O_4 + 2\ NH_4X$$

The amount of calcium precipitated is then determined by titration with hundredth normal potassium permanganate, according to the equation:

$$2\ KMnO_4 + 8\ H_2SO_4 + 5\ CaC_2O_4 = K_2SO_4 + 2\ MnSO_4 + 5\ CaSO_4 + 10\ CO_2 + 8H_2O$$

Results obtained in this way compare very favorably with calcium determinations obtained by ashing the serum.

Often, however, this method could not be used. Sometimes not sufficient sample was collected to permit separate calcium and protein determinations, but still more frequently the serum obtained was not clear, straw-colored liquid, but rather milky and cloudy. It contained some unknown suspension that could not be centrifuged out. Such a sample might be obtained from the blood of any bird, but hens in production nearly always furnish that type of serum. That difficulty of this kind is frequently encountered is indicated by the fact that bacteriologists often express difficulty in running certain precipitin tests with poultry serum. The existence of such a suspension in many samples of chicken serum is substantiated by Hughes in unpublished data, which does not establish the nature of the material, but does report that it is neither protein or lipin in character. This was partially substantiated in this research, as it was found that protein determinations on such samples ran no higher than normal.

It was practically impossible to run total serum calciums by titration on these samples, as the suspended matter precipitated along with the calcium oxalate, and interferred with the permanganate titration. After titration a white

mass remained in the tube.

In cases like this total calcium was determined by the colorimetric method of Fiske and Subbarow (32). In this method the calcium was precipitated as calcium phosphate, this precipitate dissolved in a molybdic acid solution, and the resulting phospho molybdate reduced with alpha-amino-napthol-sulphonic acid reagent. The resulting solution was then compared against a standard solution in the colorimeter, and the calcium calculated.

To determine diffusible calcium, the ultrafiltrate was obtained as previously described. Filtering at even ten atmospheres was very slow. The first two cc. usually came through in an hour and a half, but each additional cc. generally took at least one hour to filter through. If time permitted, a little more than 4 cc. of filtrate were obtained, so that duplicate 2 cc. samples could be used; otherwise only a little more than 2 cc. were filtered, 1 cc. samples being used to determine calcium. The ultrafilter was disassembled and a new filter put in between each run. Calcium was determined on all ultrafiltrates by the modified Clark and Collip method.

On most of the samples, though not in all cases, the serum protein content was determined. To do this the proteins were precipitated from the serum by diluting one volume of serum with four volumes of 10% trichloracetic

acid. The protein precipitate was then filtered free of serum on a good grade quantitative filter paper. Next it was washed several times with water made slightly acid by the addition of some of the 10% trichloracetic acid. This washing insured the elimination of the non-protein nitrogen of the blood serum. Lastly, the filter paper and contents were transferred to flasks and the nitrogen determined by the Kjeldahl method. A blank was run on the filter paper and then the protein content of the serum calculated, assuming one cubic centimeter of serum weighed one gram.

A very few determinations for diffusible calcium were made on serum from other animals. The results obtained compared favorably with similar determinations by other investigators. This indicates that the data collected in analyzing chicken serum, can be reasonably compared to the established data on mammalian blood.

RESULTS AND DISCUSSION

Data are presented on three groups of chickens; roosters, hens known not to be in production, and a group of hens each of which was known to be in active production. The division of the two groups of hens mentioned was on the basis of their ovarian activity as indicated by trap-nest records. Intermediate types, discussed later, were not used.

TABLE I

RESULTS OF ANALYSIS OF ROOSTER BLOOD.

Mg. Ca. per 100 cc. of Serum	Mg. Diffusible Ca per 100 cc. of Ultrafiltrate	Per Cent Diffusible Ca	G.'s Protein per 100 cc. of Serum
12.20	5.86	48.03	
10.54	5.27	50.00	
10.94	6.27	57.31	
10.70	6.44	60.10	
14.40	7.55	52.43	
13.50	6.15	45.55	
12.20	7.80	63.90	
10.72	5.00	46.64	39.4
11.14	6.09	54.66	48.1
12.55	5.62	44.78	
10.99	6.35	57.77	
10.74	5.73	53.35	
10.74	6.49	60.42	
13.09	7.94	57.21	
11.35	6.60	58.14	
11.05	6.44	58.29	
11.46	6.69	58.37	
Mean 11.66	6.37	54.63	
Standard Deviation ±1.11	±0.79		
Possible Error ±0.18	±0.13		

TABLE II

RESULTS OF ANALYSIS OF BLOOD FROM HENS NOT IN PRODUCTION

	Mg. Ca. per 100 cc. of Serum	Mg. Diffusible Ca per 100 cc. of Ultrafiltrate	Per Cent Diffusible Ca	G.'s Protein per 100 cc. of Serum
	12.40	6.10	49.19	
	13.20	7.55	57.19	
	11.82	6.96	58.88	45.6
	9.68	6.01	62.73	51.3
	13.15	6.85	52.09	55.0
	12.15	6.91	56.87	49.4
	11.43	7.23	63.25	36.9
	11.84	5.23	44.17	43.1
	12.83	8.30	64.69	62.5
	13.15	6.10	46.38	39.4
	11.10	5.77	51.98	
	12.80	5.92	46.25	
	11.94	6.27	52.51	
	10.60	6.47	61.04	
Mean	12.00	6.55	54.58	48.52
Standard Deviation	±1.02	±0.78		±7.68
Possible Error	±0.18	±0.14		±1.81

TABLE III

RESULTS OF ANALYSIS OF BLOOD FROM HENS IN ACTIVE PRODUCTION

Mg. Ca. per 100 cc. of Serum	Mg. Diffusible Ca per 100 cc. of Ultrafiltrate	Per Cent Diffusible Ca	G.'s Protein per 100 cc. of Serum
22.45	6.00	26.72	66.3
29.10	6.75	23.19	55.6
26.20	6.50	24.80	96.3
25.30	5.50	21.73	61.9
23.55	5.45	23.14	50.0
22.30	7.60	34.08	58.1
22.98	6.60	28.72	65.0
24.70	7.90	31.98	59.4
21.57	5.70	26.42	55.6
28.56	6.76	23.66	55.6
18.18	6.02	33.11	56.3
24.25	6.55	27.01	56.9
26.40	6.56	20.53	54.2
36.86	7.46	20.24	
18.60	6.07	32.65	
36.68	5.37	14.64	
19.15	5.97	31.17	
Mean 25.11	6.40	25.49	60.86
Standard Deviation ±5.22	±0.73		±11.08
Possible Error ±0.85	±0.12		±2.08

Table I gives the data obtained from the analysis of rooster blood. It will be seen that the total serum calcium ranges between values of 10.54 mg. as a minimum to 14.40 mg. of calcium as a maximum. The arithmetical average is 11.66 mg. $\pm$ 1.11 mg. of total calcium per 100 cc. of serum. The diffusible calcium varied from 5.00 mg. to 7.94 mg., averaging 6.37 mg. $\pm$ 0.79 mg. per 100 cc. of ultrafiltrate. Thus it is observed that from 45 to 63 per cent of the total calcium is diffusible, or on an average 54.6 per cent of the calcium in rooster serum is of the diffusible type, while 45.4 per cent is bound in such a way as to be non-filterable.

Since most of the results of the determinations of calcium in the rooster had been obtained before it was decided to also study the protein values in blood serum, only two protein determinations are shown. This paucity of values makes it impossible to compare the protein content of the rooster with the other chickens, though the similarity of these two values to those given for the non-laying hen should be observed.

Reference to Table II, giving the data from the analysis of blood of hens not in production, will show a likeness to the values just seen in Table I. The total calcium values range between 9.58 mg. to 13.2 mg. per 100 cc. of

serum, the arithmetical average being 12.0 mg. _ 1.02 mg. of calcium, or only slightly higher than the average for roosters.

The diffusible calcium values fall between 5.23 mg. and 8.30 mg., with an average of 6.55 mg. ± 0.78 mg. per 100 cc. of ultrafiltrate. Thus it will be noticed that between 44.1 to 64.6 per cent of the total calcium in the serum of this type of hen will filter through a membrane, while 45.42 per cent of the calcium is non-diffusible.

From eight determinations of the protein value in the serum, it is seen that the non-producing hen has an average of 48.52 grams ± 7.68 g. of protein per 100 cc. of serum.

The data obtained in the analysis of the blood of hens in active production are given in Table III. Here the total serum calcium values fall between the limits of 18.18 mg. to 36.86 mg., having an arithmetical average of 25.11 mg. ± 5.22 mg. per 100 cc. of serum. It will be readily noted that this value for total calcium is more than double the values obtained for the blood of roosters and non-laying hens.

The diffusible calcium values in the laying hen run from 5.37 mg. to 7.90 mg., with an average of 6.40 mg. ± 0.73 mg. per 100 cc. of ultrafiltrate. This means that in a laying hen between 14.64 to 34.08 per cent of the

total calcium is diffusible or an average of 25.49 per cent. Thus 74.51 per cent of the calcium is non-diffusible.

Protein determinations fell within the boundaries of 50.0 g. to 96.3 g. or there was an average of 60.86 g. ± 11.08 g. per 100 cc. of serum.

The results of treating the values given in Tables I, II and III statistically are given in Table IV. This establishes mathematically the places where significant differences may be assumed.

It will be noted that no significant difference is evident in the total calcium or diffusible calcium of the rooster and non-laying hen. This table also shows the interesting fact that absolutely no significant difference exists in the amount of diffusible serum calcium in the rooster, the non-laying hen, or the hen in active production.

In contrast to this, the difference of the arithmetical means of 13.45 mg. ± 0.9 mg. in total calcium between the laying hen and rooster is very significant. The laying hen shows a similar significant advantage over the non-laying hen in total serum calcium, their mean differences being 13.11 mg. ± 0.9 mg.

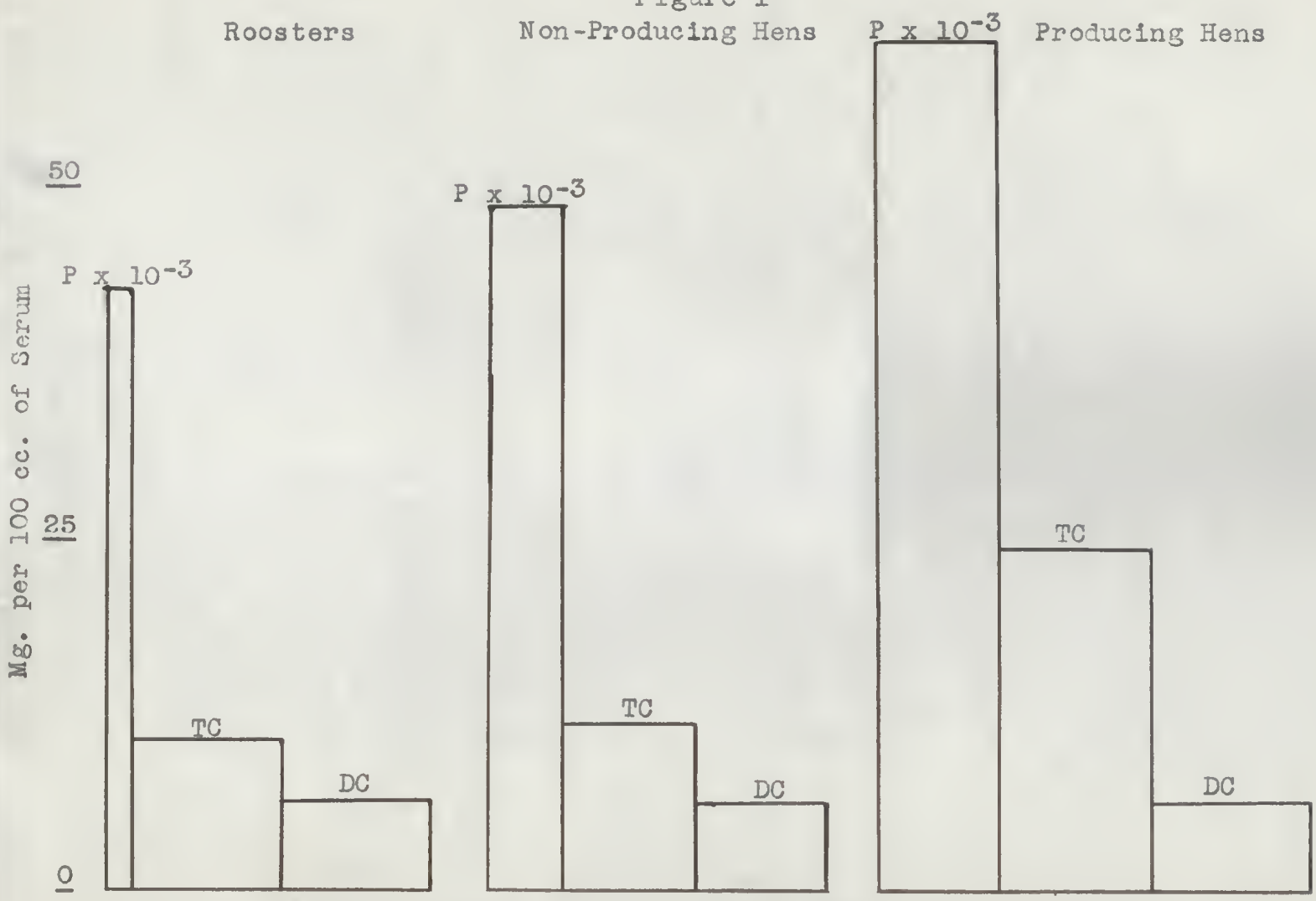
If the protein values of the laying hen and non-laying hen are compared it will be seen that the difference of their means is 12.34 g. ± 2.8 g.; this likewise seems

TABLE IV

RESULTS OF STATISTICAL COMPARISONS OF ROOSTER BLOOD, TABLE I, NON-PRODUCING HEN BLOOD, TABLE II, AND PRODUCING HEN BLOOD, TABLE III.

	Table I vs. Table II	Is Difference Significant?	Table I vs. Table III	Is Difference Significant?	Table II vs. Table III	Is Difference Significant?
Total Calcium	0.54±0.3	No	13.45±0.9	Yes	13.11±0.9	Yes
Diffusible Calcium	0.18±0.2	No	0.03±0.2	No	0.15±0.2	No
Protein					12.34±2.8	Yes

Figure 1



Width of blocks indicates number of determinations
P=Protein TC=Total Calcium DC=Diffusible Calcium

significant. No comparisons could be made with the roosters since there was not sufficient data on the protein content of their serum.

A graphic comparison of the data given in the tables is shown in Figure 1. Here again it will be noted that the total calcium and protein in the laying hen rises well above those in the rooster and non-laying hen. On the other hand the diffusible calcium values rise to the same height in each type of chicken.

This accentuates the interesting fact that no matter what state of ovarian activity, in regard to egg production, the hen may be in, she still maintains a comparatively constant amount of diffusible calcium. That is, it is the non-diffusible fraction that rises as the total serum calcium increases. This explains how a hen can be perfectly normal and yet have a calcium concentration in her blood that would be fatal in such quantities in mammalian blood.

It is interesting to note that though the highest total calcium values are in the table for laying hens, yet the highest individual value for diffusible calcium is in the table for roosters. It should also be noted that one determination for serum protein in the laying hens is up to 96.3 mg. which appears to be out of line with the other results. Without that value the average for the protein of

laying hens would be only slightly higher than that given for non-laying hens.

How this increased serum calcium is bound is undecided. As previously reviewed, most authors hold that the non-diffusible fraction is protein bound, and is in equilibrium with the diffusible calcium fraction, and by experiments in which calcium chloride was injected intravenously they concluded that the diffusible and non-diffusible calcium fractions went up together. Work of this artificial type will have to be done with roosters, and hens in different stages of ovarian activity before any conclusions can be drawn as to how the excess calcium is bound. Since the total calcium increases up to nearly 200 per cent when the protein value apparently increases no more than 6%, it can hardly be said that they increase together. There might, however, be some special small component of the serum protein which holds the non-diffusible calcium, and which increases as the total calcium increases.

As stated in introducing the tables laying hens and non-laying hens are best segregated by trap-nest records. The calcium value in hens' blood rises and falls, depending on several factors. As reviewed from Russell, Howard and Hess (27), substantiated by Hughes, the amount of calcium to be found in hens' blood is dependent upon the size of

eggs in the ovary. Thus a hen may have a comparatively high calcium value and yet just be going into or coming out of active production. This intermediate type of hen was not used as a basis of argument for this thesis; yet, as might be expected from the results presented, the diffusible calcium fraction in this type is likewise a constant, the non-diffusible calcium increasing as the total calcium goes up.

SUMMARY AND CONCLUSIONS

The arithmetical mean value of diffusible calcium of roosters was found to be 6.37 mg. $\pm$ 0.79 mg., that for non-laying hens 6.55 mg. $\pm$ 0.78 mg., and that for hens in active production, 6.40 mg. $\pm$ 0.73 mg.

These results show that the diffusible calcium in chicken serum remains constant regardless of ovarian activity.

The total calcium for laying hens was found to be two to three times as high as that for non-laying hens or roosters, which is in accord with previous reported results.

The ratio of diffusible to non-diffusible calcium does not remain constant as the total calcium in laying hens increases.

Since this ratio is said to remain constant in mammalian blood, when the total calcium is increased in any

way, it would appear that the non-diffusible calcium is held differently in the blood of laying hens from the way it is held in mammalian blood.

Additional work will be necessary to determine the nature of this bound calcium in the blood of laying hens.

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