PHOSPHORUS PARTITION IN THE BLOOD SERUM OF LAYING HENS

by

RAYMOND ROLLIN ROEPKE

B. S., Kansas State College
of Agriculture and Applied Science, 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

1934

TABLE OF CONTENTS

INTRODUCTION

The blood serum of the hen is an interesting medium for the study of mineral partition since it has been shown that the concentrations of both total phosphorus and calcium increase appreciably during the laying season (1, 2, 3). While much work has been done in recent years in an attempt to determine the nature of the non-diffusible or bound calcium in blood serum, little has been done concerning the partition of the phosphorus.

Laskowski (4) has called attention to the fact that the percentage of bound (non-ultrafilterable) inorganic phosphorus increases along with the increase of bound calcium in the serum of laying hens, and has suggested the possibility of the existence of a colloidal calcium-phosphorus complex in the serum.

This study was made in order to investigate more thoroughly the partition of the organic as well as the inorganic forms of phosphorus in the serum of laying hens.

REVIEW OF LITERATURE

Some references are given in the literature in regard

to the various phosphorus fractions in the blood and serum of human beings, calves, and chickens, but none as far as could be noted in regard to the complete partition of phosphorus in the serum of the laying hen.

Malan and Green (5) noted that in the analysis of whole blood the total phosphorus is often greater than the sum of the lipoid and acid soluble fractions. This they believed due to the presence of nucleoproteins in the red blood cells, since they found this additional fraction in the blood of young calves in which some nucleated erythrocytes were still present. Later on, as the calves grew older and the nucleated cells disappeared, the total phosphorus became equal to the sum of the lipoid and total acid soluble. This phosphoprotein also appears in an appreciable amount in the blood of chickens, since in chicken blood the erythrocytes are typically nucleated. On plasma, however, the sum of the acid soluble and lipoid phosphorus is approximately equal to the total phosphorus.

Greenwald (6) concluded that protein phosphorus is present only in small amounts in human blood, and is negligible if at all present in the serum.

Stearns and Warweg (7) found from their determinations of the phosphorus fractions of serum and blood of

children and adults that in the whole blood, as in the serum, the total phosphorus equals the sum of lipoid and total acid soluble. A table of the results of other investigators which they include in their publication tends to substantiate their conclusions.

The results of Plass and Tompkins (8), however, indicate the presence of a phosphoprotein in the maternal serum since the total phosphorus is higher than the sum of the two fractions. They also found the lipoid phosphorus to be higher than in the serum of normal women. Their results may be questioned since in several cases the lipoid phosphorus was greater than the total, and in several the inorganic phosphorus was slightly higher than the total acid soluble fraction.

EXPERIMENTAL PROCEDURE

The blood serum of laying hens, non-laying hens, and roosters was analyzed for total, lipoid, total acid soluble, inorganic, and protein phosphorus and for calcium. The ultrafiltrate of the serum was analyzed for inorganic phosphorus.

In order to obtain sufficient serum for the analysis, the chickens were bled from the heart by means of a

syringe. From 30 to 40 cc. of blood could be taken in this manner without sacrificing the bird. From 12 to 15 cc. of serum was required for the complete analysis.

The ultrafiltrates of the serum used in the analyses were obtained by the method described by Nicholas (9). Calcium was determined by the method of Roe and Kahn (10), and inorganic phosphorus by Youngburg's method (11).

Some difficulty was experienced in determining the organic forms of phosphorus by the ordinary method, that of wet ashing with sulphuric acid and hydrogen peroxide, since it is difficult to control the temperature or time of heating. Any variation in the temperature or time of heating would influence the amount of acid remaining after oxidation. Since the pH of the final solution as well as the concentration affects the intensity of color, it is important that the pH of the solution of the sample be as near as possible to that of standard when the color is developed.

Shortly after this work was started, Stearns and Harweg (7) published an article giving the results of their work in which they obtained fairly consistent results using the wet ash method by carefully heating on a hot plate. This method is no doubt quite time consuming, especially if

only a few samples are run at one time.

Green (13) obtained fairly satisfactory results by means of his method in which he neutralized the solution to phenolphthalein before developing the color. He also removed the possibility of any loss of phosphorus by dry ashing the sample after the addition of a small amount of calcium acetate which made a distinctly alkaline ash.

It was found that Green's method, while much better than the method of wet ashing ordinarily used, did not give checks as good as might be expected. This is probably due to the lack of a sharp end point with phenolphthalein when sulphuric acid is neutralized with ammonium hydroxide which is a relatively weak base.

Another objection to Green's method is that the ash contains some carbon particles even with careful heating. This necessitates filtering, which is a rather tedious process since the volume of the filtrate must be kept within 60 or 70 cc.

In the method finally used for determining the organic forms of phosphorus, the samples were dry ashed with magnesium nitrate, dissolved in sulphuric acid, and neutralized with ammonium hydroxide to congo red. By means of magnesium nitrate a perfectly white ash is ob-

tained, giving a clear solution which does not require filtering. Congo red gives a much more distinct end point than phenolphthalein since it changes at a lower pH (pH 3 to 5).

The method as developed will be given in some detail. The reagents needed are prepared as follows:

1. Magnesium nitrate solution. 50% solution of $Mg(NO_3)_2 \cdot 6H_2O$.

2. 10 N sulphuric acid. 280 cc. of concentrated sulphuric acid per liter of solution.

3. Congo red test paper. Saturate sheets of filter paper with a 1.0% water solution of congo red, and allow to dry.

4. 5 N ammonium sulphate. 330 gm. ammonium sulphate made up to one liter.

5. Alcohol-ether mixture. Three volumes 95% alcohol plus one volume ether.

6. Trichloracetic acid solution. Approximately 10% aqueous solution.

7. One per cent stannous chloride, prepared fresh each day. Dissolve 0.1 gm. of pure tin foil in 2 cc. concentrated hydrochloric acid, adding a drop of 5% copper sulphate solution and heating to aid solution. Dilute to

approximately 10 cc. with cold water and filter.

8. Molybdic-sulphuric acid reagent (Denige's reagent). Dissolve ten gm. of ammonium molybdate in 100 cc. water, filter if necessary. Mix 150 cc. concentrated sulphuric acid with 150 cc. of water. Cool and mix the two solutions.

9. Standard phosphorus solution. Dissolve 0.4389 gm. pure, dry monopotassium phosphate in water sufficient to make 1000 cc. 1.0 cc. = 0.1 mg. of phosphorus. Add a few drops of chloroform to prevent mold formation. For the working standard dilute 10 cc. of the stock solution to 100 cc. 1 cc. = 0.01 mg. of phosphorus.

The procedure followed in the analysis is given as follows:

Total phosphorus. Into a 150 cc. beaker pipette an aliquot of diluted serum containing from 0.05 to 0.15 mg. (preferably about 0.10 mg.) of phosphorus. Add 2 cc. of the magnesium nitrate mixture and evaporate on hot plate, increasing the heat until a considerable part of the ash has become white, 15 to 30 minutes at the highest temperature is sufficient. If a portion of the ash becomes charred it will be necessary to add several cc. of water and a drop of nitric acid and repeat the heating. When

dry, the sample is heated over a free flame (or in a muffle) until all of the magnesium nitrate has been decomposed, as evidenced by the absence of brown, nitrous oxide fumes. This should leave a perfectly white ash. If an appreciable amount of carbon remains, it must be oxidized by adding a small amount of water and a drop of nitric acid, and reheating. The ash is dissolved in 2.5 cc. of 10 N sulphuric acid and 10 to 20 cc. of water, heating to insure complete solution. Cool, add a small piece of congo red test paper, and neutralize with concentrated ammonium hydroxide. Transfer the solution to a 100 cc. volumetric flask through a short stemmed funnel containing a bead or some other form of constriction which will retain the test paper. Wash the beaker and funnel thoroughly, preferably with hot water, and add sufficient water to bring the volume to 70 to 80 cc. To both standard and unknown add from a burette 2 cc. of the molybdic-sulphuric acid reagent, mixing the solution by shaking before and after adding the reagent. Dilute to 100 cc., add 0.5 cc. of the stannous chloride solution and mix immediately. After one or two minutes compare in a colorimeter, setting the standard at 30.

Lipoid phosphorus. Into a 50 cc. volumetric flask

containing about 40 cc. of the alcohol-ether mixture, add 2 cc. of serum, mix, and heat to boiling. Cool, make up to the mark with the alcohol-ether mixture, and filter. Pipette 10 to 20 cc. of the filtrate into a 150 cc. beaker, add 1 cc. of magnesium nitrate solution, and proceed as for total phosphorus.

Total acid soluble phosphorus. To one volume of serum add four volumes of 10% trichloracetic acid, mix, centrifuge, and filter. Pipette 5 to 10 cc. of filtrate into a small beaker, add 1 cc. of magnesium nitrate solution and proceed as for total phosphorus. Care should be taken to see that all of the trichloracetic acid has been volatilized by heating on the hot plate before heating over a free flame, since too rapid heating will cause it to char.

Protein phosphorus. To 5 cc. of serum in a centrifuge tube add 20 cc. of 10% trichloracetic acid, centrifuge until the supernatant liquid is clear, and decant. Wash the precipitate three or four times with 10% trichloracetic acid, and three or four times with a hot mixture of alcohol-ether. Transfer the precipitate to a small beaker, add 3 or 4 cc. of magnesium nitrate, and proceed as for total phosphorus except that an aliquot

should be taken in cases of high phosphorus. It will also be necessary to dissolve the sample in more than 2.5 cc. of the 10 N sulphuric acid, and add an equivalent amount of ammonium sulphate to the standard.

In this procedure the ammonium sulphate is added to the standard to compensate for the ammonium sulphate formed in the solution of the sample. The presence of ammonium sulphate produces a buffer effect which leaves the pH of the solution after the addition of the molybdic-sulphuric acid reagent higher than it would be if no ammonium sulphate were present. Therefore in order that the pH of the two solutions be equal, approximately the same amount of the salt must be present in the two solutions.

The concentration of the standard should be chosen so that the reading of the sample will be within three or four units of the standard, when the standard is set at 30. Unless the approximate concentration of phosphorus in the sample is known a series of standards should be made up for a number of samples. The molybdic-sulphuric acid reagent should be added to all the solutions of a series and the solutions mixed and diluted before stannous chloride is added, since the blue color begins to fade soon after it

has been developed.

RESULTS AND DISCUSSION

Data are presented on three groups of chickens: roosters, laying hens, and non-laying hens. The separation of the hens into laying and non-laying groups is based chiefly on the concentration of calcium in the serum rather than on their egg production record. Whenever possible the laying hens were selected from those on the nest about to lay. However, there are, no doubt, several hens included in the group of laying hens whose egg production is quite low. More will be said later concerning these.

From Table I it can be seen that the results of the analyses of the serum of roosters and non-laying hens conforms very well with the results obtained by other investigators on various serums in that the total phosphorus is equal to the sum of lipoid and total acid soluble phosphorus. There is apparent, however, a tendency for the total phosphorus to be slightly less than the sum of the lipoid and total acid soluble phosphorus, indicating a slight over-lapping of the two fractions.

The most noticeable difference between the serum of

laying hens and roosters, as seen in Table II, is the appearance of a new fraction of phosphorus. The total phosphorus in the serum of laying hens is much greater than the sum of lipoid and acid soluble phosphorus. This can best be explained by the existence of a phosphoprotein in the serum. The protein phosphorus as determined agrees fairly well with the difference between the total phosphorus and the sum of lipoid and acid soluble fractions as is shown in Table II. Another noticeable difference is the large increase in lipoid phosphorus in the serum of the laying hen over that in rooster serum.

An attempt was made to determine the nature of the phosphoprotein in the serum of laying hens. This was done by subjecting it to acid and pepsin hydrolysis and to immunological tests. The proteins in 5 cc. of serum was precipitated with trichloracetic acid, extracted with trichloracetic acid and hot alcohol-ether mixture, and treated with different concentrations of acid under varying conditions of time and temperature. The protein was then reprecipitated by the addition of solid trichloracetic acid and the filtrate analyzed for phosphorus. The alcohol-ether extract of the reprecipitated protein gave no test for phosphorus under any condition of hydrolysis,

TABLE I

RESULTS OF ANALYSIS OF BLOOD SERUM OF ROOSTERS AND NON-LAYING HENS

(Results expressed as mg. per 100 cc.)

	Serum Phosphorus							
					Total-(Lipoid + Acid Sol.)	Inorganic		
	Total	Lipoid	Acid Sol.	Protein		Inor-ganic	Ultra-filterable	Calcium
Roosters								
1	14.16	7.08	6.66	-	0.42	-	-	-
2	14.92	7.65	7.35	-	-0.08	-	-	-
3	13.34	7.17	6.15	-	0.02	5.58	4.65	10.85
11	12.42	7.82	4.78	-	-0.18	4.91	2.98	10,51
12	9.55	5.17	4.05	-	0.33	4.31	2.64	10.66
13*	16.10	10.20	6.12	-	-0.22	5.77	3.85	12,13
15**	17.47	10.30	7.40	-	-0.23	6.39	4.46	12,13
19	12.08	6.77	5.64	0.4	-0.33	5.33	3.43	-
20	11.74	6.44	5.85	0.0	-0.55	5.38	3.32	-
34	8.14	5.22	3.52	-	-0.60	-	-	-
Average	-	-	-	-	-	5.38	3.61	-
Non-laying Hens								
7	15.32	10.38	2.50	-	2.44	2.16	-	12.52
16	9.15	3.78	5.12	-	0.25	5.35	3.88	11.50
28	9.37	5.95	3.80	0.25	-0.38	3.12	2.14	10.91
29	11.98	6.75	5.12	0.93	0.11	4.52	2.73	12.89
Average	-	-	-	-	-	4.33	2.91	-

* Sick rooster. Died after 25 cc. of blood had been drawn.

** Country-fed rooster, getting green feed, etc.

TABLE II

RESULTS OF ANALYSIS OF BLOOD SERUM OF LAYING HENS

(Results expressed as mg. per 100 cc.)

	Phosphorus							
	Total	Lipoid	Acid Sol.	Protein	Total-(Lipoid + Acid Sol.)	Inorganic	Inorganic Ultrafilterable	Calcium
4	40.36	23.62	5.35	-	11.39	4.90**	-	-
5	32.34	18.22	5.14	-	8.98	5.13	2.56	-
6	28.54	15.31	5.10	-	8.13	4.50**	-	16.76
8	34.78	21.76	4.41	-	8.61	4.16	1.86	19.47
9	43.69	26.95	4.47	-	12.27	3.95	1.46	20.00
10	30.97	15.62	5.34	-	10.01	5.23	2.08	15.68
14	36.78	20.36	6.01	10.78	10.41	5.76	2.18	-
17*	45.44	29.60	4.77	11.78	11.07	3.98	1.35	22.04
18*	42.85	25.16	5.49	12.16	12.20	5.52	1.38	19.00
22	35.28	20.90	6.42	7.50	7.96			
23*						3.75	1.37	19.13
25*						4.38	1.91	17.86
30	21.82	12.63	4.84	3.58	4.35	4.41	1.36	19.71
31	33.46	17.70	7.01	-	8.75	6.55	2.15	23.46
32	31.80	14.88	6.89	10.41	10.03	6.45	2.03	23.52
Av.						4.94	1.80	

* Serum pooled from two or more hens.
** Not included in average.

the hydrolyzed phosphorus appearing in the acid soluble fraction.

It was found that after treating with one normal hydrochloric acid for 2 3/4 hours in boiling water bath, about 73.3 per cent of the protein phosphorus was hydrolyzed; after four hours, 85.0 per cent; and after six hours, 91.0 per cent. The protein precipitated from laying-hen serum dissolved entirely in concentrated hydrochloric acid and, after standing 3 1/2 hours at room temperature, only 25.5 per cent of the protein phosphorus was hydrolyzed. This is evidence that the phosphorus is not just adsorbed to the protein but must be bound quite firmly in the molecule.

An attempt was made to hydrolyze the phosphoprotein by means of pepsin, but it was found that although the bulk of precipitated protein was much less in the pepsin-hydrolyzed serum than in the raw serum the protein phosphorus fraction remained practically unchanged. The serum was hydrolyzed by adding to 5 cc. of serum 0.1 cc. of concentrated hydrochloric acid, 0.05 gm. of powdered pepsin, and incubating at 40°C. It was found that unless the serum had stood several days it was necessary to heat the sample at 65° to 70°C. for one-half hour before adding

the pepsin and acid in order to destroy the anti-enzymes.

It was noticed that the action of pepsin and hydrochloric acid on the phosphoprotein of hen serum agrees in general with the properties given by Jukes and Kay (14) for vitellin. The phosphorus of vitellin is held rather firmly in the protein, and is not readily hydrolyzed by hydrochloric acid at ordinary temperatures. If vitellin is subjected to pepsin hydrolysis, there remains a pepsin-resistant fraction which contains iron as well as phosphorus, the two being present in the ratio of one part of iron to 18 to 19 parts of phosphorus (15, 16, 17).

When further analyses were made on the serum of the laying hen, iron was also found to be present in the pepsin-resistant fraction but in the ratio of one part of iron to 9 to 19 parts of phosphorus. These results are given in Table III. The iron determinations may be questioned some, however, since even the same sample did not give consistent results as can be seen from the results of samples 34 and 35.

In order to obtain a comparison, a test for iron was made on the pepsin-resistant protein of rooster serum. As can be seen from Table III, iron was found to be present to the extent of from one-third to one-half of that found

TABLE III

RESULTS OF THE ANALYSIS OF THE PEPSIN-RESISTANT FRACTION OF SERUM PROTEIN

Sample No.	Hydrolyzed with Pepsin at 40°C. for:	Iron mg. per 100 cc.	Phosphorus mg. per 100 cc.	P/Fe
Laying Hens				
26	14 hr.	0.439	4.06	9.2
33	7 hr.	0.616	6.73	10.9
34	2 hr.	0.441	7.69	17.4
"	5½ hr.	0.582	7.59	13.0
"	8 hr.	0.636	7.52	11.8
35	8 hr.	0.640	12.63	19.7
"	8 hr.	0.851	12.63	14.8
Roosters				
27	8 hr.	0.25	0.62(raw	2.4
37	14 hr.	0.23	- serum)	-

in laying-hen serum. A test was made on the pepsin used and an appreciable amount of iron found to be present. However, since pepsin is soluble even in trichloracetic acid, it is doubtful whether the iron is from this source, although some of the iron may have been held by the protein even after the trichloracetic acid extractions. Since rooster blood has a tendency to lake very readily, a part of the iron may be from haemoglobin although no red color was noticed in the serum.

Since the properties of the phosphoprotein of the hen's serum compared so closely to those of vitellin, the two proteins were further compared immunologically. One rabbit received seven intravenous injections of laying-hen serum in doses of 0.1 cc. Another rabbit received equivalent doses of rooster serum. On the tenth day after receiving the last dose, both rabbits were bled and the serums tested for precipitins against a solution of egg-yolk protein with the results shown in Table IV.

The egg-yolk protein was prepared according to the directions as given by Plimmer and Scott (18) for the preparation of vitellin with the exception that the protein was not extracted with alcohol and dried, since the alcohol treatment would denature the protein so that it could not be

TABLE IV

RESULTS OF IMMUNOLOGICAL TESTS

Anti-serum No. I. Serum of rabbit injected with hen serum
Anti-serum No. II. " " " " " rooster serum
Anti-serum No. III. " " " " " egg-yolk protein
Anti-serum No. IV. " " " " " " " "

Antigen: Egg-yolk protein in 10% NaCl

Dilution of antigen	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640
Anti-serum No. I	++++	++++	++++	++++	++++	+++	+	-
Anti-serum No. II	-	-	-	-	-	-	-	-

Antigen: Rooster and laying-hen serum diluted with 0.9% NaCl

Dilution of antigen	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Anti-serum No. III									
Laying-hen serum No. 1	++++	++++	++++	++++	++++	+++	-	-	-
" " " No. 2	++++	++++	++++	++++	++++	++	-	-	-
Rooster serum No. 1	++++	++++	+++	?	-	-	-	-	-
" " No. 2	++++	++++	++++	++	-	-	-	-	-
Anti-serum No. IV									
Laying-hen serum No. 1	++++	++++	++++	++++	++++	+++	++	-	-
" " " No. 2	++++	++++	++++	++++	++++	++++	?	-	-
Rooster serum No. 1	++++	++++	+++	+	-	-	-	-	-
" " No. 2	++++	++++	+++	+	-	-	-	-	-

redissolved.

The solution of antigen used in testing the anti-serum for precipitins was made by weighing out two gms. of the centrifuged, undried egg-yolk protein and dissolving in 10 cc. of a 10% salt solution. This is equivalent to about a 1.5% solution with respect to purified vitellin. A sufficient amount of salt was added to the anti-serum to make a 10% solution. The precipitin tests were made in small glass tubes about one inch long and with an internal diameter of 1.5 to 2.0 mm. The anti-serum was put in the lower half of the tube by means of a long pointed tube; the upper part of the tube was filled with the antigen. The appearance of a white precipitate at the junction of the two liquids indicated a positive test.

In order to check the results, rabbits were immunized with egg-yolk protein and the anti-serum tested with laying-hen and rooster serum. The protein solution injected into the rabbits was made by weighing out seven gms. of the partially purified, centrifuged vitellin and adding sufficient 10% salt solution to make 10 cc. of solution. This is equivalent to about a 4.8% solution of purified vitellin. Seven doses of 0.2 cc. each were injected intravenously. The results are also shown in Table IV.

The results of these immunological tests tend to show that there is present in the serum of laying hens and roosters a protein which is similar to a protein found in the egg yolk. This protein is probably vitellin although further work must be done before any definite conclusions can be made.

According to the results of Osborne and Campbell (19), vitellin contains 0.94 per cent phosphorus. Thus if all of the protein phosphorus of the hen's serum is in the form of vitellin, the serum of the laying hen would contain about one per cent vitellin, or in other words vitellin would constitute at least one-fifth of the total serum protein. Not withstanding this, the average concentration of protein in the serum of laying hens is no greater than the average in rooster serum. In fact an analysis of two lots of serum pooled from ten gave an average of 4.7 per cent protein, while an analysis of two lots of pooled serum of roosters gave an average of 5.1 per cent protein as determined by the Kjeldahl method.

The vitellin of the egg yolk is said to be the source of haemoglobin for the young chick. Most of the iron of the egg yolk is found to be combined in the form of protein (14), and Plimmer and Scott (18) have observed that about

the fourteenth day of incubation there is sudden decrease in the vitellin content of the egg yolk along with a rapid increase in the haemoglobin content of the chick. Hugounerq and Morel (20) noted that the pepsin-resistant fraction of vitellin (haematogen) could be treated to give substances which resembled hematin and its derived pigments.

Since there seems to be a close relation between the vitellin of the egg yolk and the haemoglobin of the young chick, it seems likely that the vitellin-like protein of the hen's serum might be derived by the addition of inorganic phosphorus and perhaps some protein fraction to an iron-containing protein envolved in the normal formation of haemoglobin. This appears more likely when it is noted that there may be an iron-containing protein even in rooster serum, and that the phosphoprotein may constitute one-fifth of the amount of protein in the serum of the laying hen, although the total amount of protein is no greater than that in rooster serum.

Knowing that the yolk of the egg contains a large amount of phosphoprotein (vitellin) and of lecithin, it is not at all surprising that some form of phosphoprotein makes its appearance in hen serum or that the lipoid

phosphorus is much higher in the serum of laying hens.

If the phosphoprotein of the hen's serum is the precursor of the vitellin in the egg yolk, the manner in which it is transferred from the serum to the yolk is difficult to understand. Whether the protein is transferred unchanged to the yolk or whether it suffers disintegration in the serum with its fractions resynthesized into vitellin in the yolk is yet to be determined.

By comparing the lipoid phosphorus with the calcium in Table II, it appears that the concentration of lipoid phosphorus in hen serum may be a better indicator of egg production than the amount of serum calcium. It can be seen that as the calcium increases in the serum the lipoid phosphorus also is higher, the two being present in somewhat of a constant ratio with the exception of hens 30, 31, and 32. These hens, along with 28 and 29, were received from a local poultry firm on June 9 as non-producing hens. They were immediately put on a feed consisting of laying mash and oyster shell. After five days, samples of blood were drawn and analyzed.

As is shown in Table II, the serum calcium of these three hens is as high as that of normal laying hens although the lipoid phosphorus is much lower. No eggs were

laid by these hens during the first week of observation and only four in the second week. Apparently the hens were just going into production.

The results of the ultrafilterable phosphorus in Tables I and II at first appear to disagree with those of Laskowski (4), but with further study it can be seen that they agree quite closely in so far as the relative amount of ultrafilterable phosphorus is concerned.

Laskowski found that average concentration of inorganic phosphorus in the blood plasma of non-laying hens was 2.9 mg. per 100 cc.; and in the ultrafiltrate, 2.8 mg. per 100 cc., or in other words 97 per cent of the inorganic phosphorus was ultrafilterable. In the plasma of the laying hen, he found an average of 4.1 mg. of inorganic phosphorus per 100 cc. of plasma, and 2.2 mg. per 100 cc. of ultrafilterable, or that 54 per cent of the inorganic phosphorus of plasma was ultrafilterable. From Table I the inorganic phosphorus of both rooster and non-laying-hen serum is found to be 67 per cent ultrafilterable, and that of laying-hen serum to be 36.5 per cent ultrafilterable. In other words the percentage of ultrafilterable phosphorus in hen serum is only slightly over half of that of rooster serum. This agrees with the results of Laskowski, since

his results show the percentage of ultrafilterable phosphorus in the plasma of laying hens to be about one-half that of the plasma of roosters.

The two results differ in that Laskowski found a higher percentage of ultrafilterable phosphorus in each. However, this may be explained, as Laskowski has indicated, by the fact that he obtained his ultrafiltrates under reduced pressure. This would mean that the filtrates would be concentrated by evaporation, but since he used membranes of approximately the same rate of filtration his results are comparable to each other.

The results of the ultrafilterable phosphorus also agrees quite well with those of Benjamin and Hess (21) with respect to rooster serum but not with respect to the serum of laying hens. They found the inorganic phosphorus to be 6.1 mg. per 100 cc. in rooster serum and 4.3 in the ultrafiltrate. In other words they found the inorganic phosphorus to be 72 per cent ultrafilterable. In laying-hen serum they found 4.0 mg. out of 6.2 mg. or 64.5 per cent of the inorganic phosphorus to be ultrafilterable. This is not in agreement with the results of this work or with those of Laskowski. The difference may be due to the high inorganic phosphorus content in the serum of the

chicken, indicating a high phosphorus feed which may affect the percentage of ultrafilterable phosphorus in the hen serum.

Laskowski (4) has suggested that the non-ultrafilterable inorganic phosphorus has formed a colloidal, calcium-phosphorus complex which would account for the large amount of bound calcium in the serum of the laying hen. It is difficult to see how this relatively small amount of phosphorus could combine with such a large amount of calcium.

Since the amount of lipoid and protein phosphorus increases along with the increase in calcium in the laying hen, there may be some relation between those organic forms of phosphorus and the bound calcium. More work will have to be done on this problem before any definite conclusions can be made.

SUMMARY AND CONCLUSIONS

The total phosphorus in the serum of roosters and non-laying hens was found to be approximately equal to the sum of lipoid and acid soluble phosphorus.

The total phosphorus in the serum of laying hens was found to be from 4 to 12 mg. per 100 cc. higher than the

sum of the lipoid and acid soluble phosphorus.

This indicates the presence of a phosphoprotein in laying-hen serum which is almost negligible if at all present in the serum of roosters and non-laying hens.

A protein in the serum of laying hens and roosters was found to be immunologically similar to a protein in the egg yolk.

The lipoid phosphorus of roosters and non-laying hens varies from four to ten mg. per 100 cc. while that of laying hens varies from 13 to 30 mg. per 100 cc.

The percentage of inorganic phosphorus which was found to be ultrafilterable in the serum of laying hens is about one-half of that in the serum of roosters and non-laying hens. This is in agreement with results reported by other investigators.

Additional work will be necessary to determine the relation between the increase of lipoid and protein phosphorus and the bound calcium in the serum of the hen.

ACKNOWLEDGMENTS

The author wishes to take this opportunity to express his sincere appreciation to Dr. J. S. Hughes for his suggestions and assistance in working out this problem, to Dr. C. H. Whitnah for his suggestions, and to Dr. L. D. Bushnell and Helen McCord of the Department of Bacteriology for their assistance in conducting the immunological tests.

LITERATURE CITED

(1) Hughes, J. S.
Mineral analysis of blood of laying hens and mature roosters. In Kan. State Agr. Exp. Sta. Director's Report, 104-5. 1926-1928.

(2) Hughes, J. S., Titus, R. W., and Smith, B. L.
Increase in the calcium of hens' blood accompanying egg production. Science 65: 264-66. March, 1927.

(3) Correll, John T., and Hughes, J. S.
The relation of filterable to non-filterable calcium in chicken blood. Jour. Bio Chem. 103: 511-14. Dec. 1933.

(4) Laskowski, M.
Uber den Calciumgustard im blut Plasma der Henne. Biochem. Zeit. 260: 230-40. April, 1933.

(5) Malan, A. I., and Green, H. H.
The unknown phosphorus fraction of calf blood. Jour. Agr. Sci. 18: 391-95. July, 1928.

(6) Greenwald, Isador.
The estimation of lipoid and acid soluble phosphorus in small amounts of serum. Jour. Bio Chem. 21: 29-36. May, 1915.

(7) Stearns, Genevieve, and Warweg, Edna.
The partition of phosphorus in whole blood and serum, the serum calcium, and plasma phosphatase from birth to maturity. Jour. Bio Chem. 102: 749-65. Oct. 1933.

(8) Plass, E. D., and Tompkins, Edna H.
The various phosphoric acid compounds in maternal and fetal serum. Jour. Bio Chem. 56: 309-17. June, 1923.

(9) Nicholas, H. O.
Diffusible serum calcium by high pressure ultrafiltration. Jour. Bio Chem. 97: 457-63. Aug. 1932.

(10) Roe, Joseph H., and Kahn, Bernard S.
The colorimetric determination of blood calcium. Jour. Bio Chem. 81: 1-7. Jan. 1929.

(11) Youngburg, Guy E., and Youngburg, Mamie V.
Phosphorus metabolism. A system of blood phosphorus analysis. Jour. Lab. Clinc. Med. 16: 158-66. Nov. 1930.

(12) Truog, Emil, and Meyer, H. H.
Improvements in the Denige's colorimeter method for phosphorus and arsenic. Ind. Eng. Chem. Anal. Ed. 1: 136-39. July, 1929.

(13) Green, H. H.
Determination of phosphorus compounds in blood by dry combustion. Jour. Agr. Sci. 18: 372-75. July, 1928.

(14) Jukes, T. H., and Kay, H. D.
Editorial review. Egg-yolk proteins. Jour. Nutr. 5: 81-101. Jan. 1932.

(15) Bunge, G. von.
Zeit. Physiol. Chem. 9: 49. 1882. As cited by Jukes and Kay (14).

(16) Hugounerq, L., and Morel, A.
Jour. de Physiol. et Path. Gen. 8: 391. 1906. As cited by Jukes and Kay (14).

(17) Hugounerq, L., and Morel, A.
Recherches sur l'hematogene. Compt. Rend. 140: 1065-67. April, 1905.

(18) Plimmer, R. H. Aders, and Scott, F. H.
A reaction distinguishing phosphoprotein from nucleoprotein and the distribution of phosphoproteins in tissues. Jour. Chem. Soc. 93: 1699-721. 1908.

(19) Osborne, T. B., and Campbell, G. F.
The proteids of the egg yolk. Jour. Am. Chem. Soc. 22: 413-22. July, 1930.

(20) Hugounerq, L., and Morel, A.
Recherches sur la formation de l'hemoglobine chez l'embryon. Compt. Rend. 141: 848-50. Nov. 1905.

(21) Benjamin, Helene R., and Hess, Alfred F.
A comparison of physiological and experimental hypercalcemia. Jour. Bio Chem. 103: 629-41. Dec. 1933.