

## CLXXVIII. STUDIES IN EMBRYONIC MORTALITY IN THE CHICK.

### I. THE EFFECT OF DIET UPON THE NITROGEN, AMINO-NITROGEN, TYROSINE, TRYPTOPHAN, CYSTINE AND IRON CONTENT OF THE PROTEINS AND ON THE TOTAL COPPER OF THE HEN'S EGG.

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WHILE it is an undoubted fact that the successful hatching of a fertile egg is influenced by genetic inheritance and by the physical factors involved in the process of incubation itself, it has not been so definitely established that factors in the diet of the hen play an important part in embryonic mortality.

Our attention was drawn by Graham and Smith [1929] to the fact that possibly the solution of many of the problems of incubational mortality lay in the realms of physiological chemistry rather than in morphology. For some years past they have studied on an extremely comprehensive scale the influence of the diet of the parent fowl on egg production and on the hatchability of the eggs. Their results, of the incubation under standard conditions of over forty thousand eggs from hens of the same breeding and under controlled environmental conditions, show conclusively that the diet of the laying hen, particularly in regard to the supply of fat-soluble vitamins and the source of protein, has a profound influence both on the extent and the distribution of the mortality during the period of incubation. This dietary effect is pronounced apart from the apparent influence of individual constitutional vigour.

It is also apparent from their results that the influence of diet on hatchability is particularly pronounced during the months of February, March and April and not so apparent during May, June and July. It is to be anticipated that a deficient diet would have its greatest effect on hatchability during the mid-winter months when constitutional vigour is probably at its lowest ebb.

One of the most important generalisations that can be made as the result of modern investigations in avian nutrition is that it is not possible to secure a diet composed entirely of cereal grains and grain by-products which will promote normal growth and reproduction over any considerable period, and

that it is necessary to supplement with suitable proteins. The results of Graham and Smith show that so far as embryonic mortality is concerned it is not a matter of indifference whether the vegetable basal ration be supplemented with equal amounts of crude protein from either buttermilk powder, fish meal, cod-liver meal, meat meal or tankage.

In the winter months the mortality during incubation was greatest where meat meal, and particularly tankage, was fed as the only protein supplement. In the case of the latter the addition of cod-liver oil made very little difference. The least mortality was found where buttermilk powder was fed, especially when cod-liver oil or ultra-violet irradiation was given in addition. The feeding of the two abattoir by-products, meat meal and tankage, was found particularly to increase the embryonic mortality during the early period of incubation (1 to 6 days), this effect being most pronounced in the winter hatches. It was found that a considerable number of these embryos at three to four days development were in an anaemic condition, this being particularly prevalent in the embryos from the tankage diet.

It would appear that the numerous factors affecting the hatchability of hen's eggs have a far greater significance than is indicated by the meagre literature dealing with the problem. The investigations reported in this paper were initiated to determine whether any relationship existed between the chemical composition and the hatchability of eggs laid by hens on diets differing only in the source of protein concentrate fed. It was hoped that some light might be thrown on the dietary factors responsible for incubational mortality.

#### EXPERIMENTAL.

In the experiments conducted by Graham and Smith [1929] mature pullets, which had been raised under range conditions and which had previously been receiving the regular plant diet, were placed on the experimental diets. The influence of a diet on hatchability would, undoubtedly, be more marked should the birds used be fed on it from the time of hatching. Our endeavour was to determine the influence on the hatchability and chemical composition of the eggs from chicks fed from the time of hatching on diets which varied, as far as was possible, only in the source of protein supplement.

The Barred Rock chicks used for the experiment were grown in the Brooder house of the Poultry Department where each group had access to an outdoor concrete run which was completely screened in so that the chicks got an abundance of sunlight without contaminating the food supply. When the chicks were four months old ten pullets from each of the groups were removed to four specially constructed pens (28, 29, 30, 31) in the laboratory. The pens were constructed so that the birds were on wire, thus preventing access to their faeces, and so that trap-nests could be provided. The laboratory was heated, and the pens were situated so that they received no direct sunlight. Some minor modifications were made in the diet on which the birds had previously subsisted in an endeavour to make the only variable, as nearly as possible, the quality of

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the protein from the different protein supplements. The birds in each group, however, continued to receive the same source of protein supplement as before. The fish meal, meat meal and tankage groups received in each case an amount of the protein concentrate which added 6 % crude protein ( $N \times 6.25$ ) to the diet. The low protein content of buttermilk powder made it impossible to incorporate an equivalent amount of crude protein to that added in the other diets and still keep the remainder of the diet the same in each case. Pen 28, therefore, received the equivalent of only 3 % of crude protein from buttermilk powder. The amount of mash containing 10 % crude protein was kept the same in each pen by varying the amount of dextrin. The amount of bone ash was varied to keep the total ash content of each diet approximately the same. The composition of the diets was as follows.

Table I. *Percentage composition of the diets.*

	Pen 28	Pen 29	Pen 30	Pen 31
Buttermilk powder	10.5	—	—	—
Fish meal	—	7.9	—	—
Tankage	—	—	10.7	—
Meat meal	—	—	—	10.5
Mash*	83.5	83.5	83.5	83.5
Cod-liver oil	2.0	2.0	2.0	2.0
Bone ash	3.5	3.1	2.5	2.5
Salt	0.5	0.5	0.5	0.5
Dextrin	—	3.0	0.8	1.0

\* Ground yellow corn 70 parts; ground oats 30 parts; wheat middlings 50 parts; alfalfa meal 15 parts.

The birds commenced laying in January 1929 and the experiment was continued until August 30th, 1929. All the eggs laid, excepting those taken in the early summer for chemical studies were incubated and accurate incubation records kept. The results are given in Table IA.

Table IA. *Results of incubation trials.*

	Eggs set	Dead embryos	Infertile eggs	Chicks hatched	% of fertile eggs hatched
Pen 28. Buttermilk powder	496	213	163	120	36.0
29. Fish meal	633	318	75	240	38.4
30. Tankage	224	105	119	0	0
31. Meat meal	451	262	73	116	30.6
14. Cod-liver meal	676	91	167	418	82.0

The one male in each pen for the purpose of this part of the work was shifted to the next pen circularly each twenty-four hours so as to eliminate the fertility factor from this source as thoroughly as possible. The results of a hatchability study with cod-liver meal as the protein supplement are also included. These results (Pen 14) are not strictly comparable with those from the other pens, for while they represent the results from an equal number of pullets and at the same period of the year, the birds were not put on the experimental diet until four months before the hatching data were collected.

The cod-liver meal used had been freed by extraction from the large amount

of cod-liver oil which it contains. To do this a large copper extractor [Bryant, 1929] with a capacity of twenty pounds of meal was constructed on the Soxhlet principle, and the cod-liver meal extracted until fat-free. At first ether was used as the solvent, but later a distillate of solvent naphtha, all that distilled over under 90°, was used. The extracted cod-liver meal was then dried in the electric oven at a temperature sufficiently high to drive off all the solvent. The results represent the supplementary value of 3 % crude protein ( $N \times 6.25$ ) from oil-free cod-liver meal. These results are included at this time because of our particular interest in what influence this material might have on the hatchability of the eggs and because eggs from this diet were included in our analytical studies.

In Fig. 1 is shown the percentage distribution of the embryo mortality grouped into seven three-day periods.

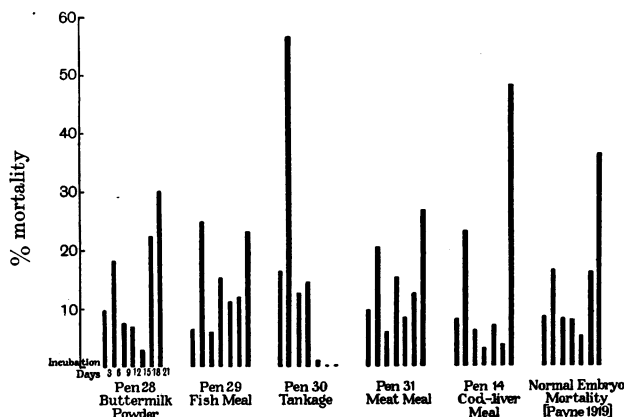


Fig. 1. The distribution of embryo mortality during incubation shown as 7 three-day periods.

The poor hatchability of the eggs from Pens 28–31 inclusive was somewhat surprising. It is of interest to compare these results with those of Graham and Smith [1929] where the same protein supplements, plus cod-liver oil, were used, but where mature pullets were placed on the experimental rations and where the conditions were such that the birds received a considerable amount of sunlight. The average results for February, March and April for both 1928 and 1929 in this experiment were as follows:

Pen	Supplement	% of the fertile eggs which hatched
4.	10 % buttermilk powder and cod-liver oil	69.5
28.	10 % fish meal and cod-liver oil	55.1
18.	10 % meat meal and cod-liver oil	67.4
30.	10 % tankage and cod-liver oil	38.7

While the results of these two experiments are not strictly comparable, they leave little doubt as to the effect which confinement of the birds and limitation

of their diet throughout growth and reproduction has on the hatchability of the fertile eggs.

The supplementary value of 3 % crude protein from buttermilk powder, so far as hatchability is concerned, was practically the same as that of 6 % crude protein from fish meal or meat meal. For some reason a very considerable number of the eggs from the buttermilk powder were infertile, which resulted in a lower hatch. Complete failure to hatch any of the eggs from the tankage group is noteworthy. The excellent hatch obtained from Pen 14, receiving fat-free cod-liver meal, would suggest that the inclusion of this meal in the diet of the laying hen has considerable value in improving the hatching quality of the eggs. Since the careful extraction of this meal with a fat solvent had removed the fat-soluble vitamins it would appear that the protein or inorganic constituents of cod-liver meal are of particular value in producing eggs of high hatchability.

The distribution of embryo mortality would also appear to vary with changes in the diet. That found with cod-liver meal and buttermilk powder is very similar to the "normal" distribution of embryo mortality during artificial incubation as described by Payne [1919]. In the meat meal and fish meal groups the embryo mortality is much more evenly distributed throughout the incubation period and, while showing the same sharply defined peaks around the 4th and 19th days of incubation as those described by Payne, there is also another critical period about the 12th day of incubation. In the case of the tankage group 56.5 % of the embryos died during the 4th, 5th and 6th days and only one embryo survived the 10th day of incubation.

Associated with this high mortality in the early period is the fact that the embryos were found in an extremely anaemic condition. On the 4th day of incubation many of the eggs from the tankage and meat meal groups, particularly the former, when candled, showed a shadow to one side of the germ spot. Such eggs were removed from the incubator, opened in warm saline and the embryo examined under the microscope. The embryo generally showed retarded development, and, while the heart beat was apparently normal, the circulatory fluid showed a lack of haemoglobin (pale) when compared with the normal, in many cases being almost colourless. Over 50 % of the embryos from the tankage diet were anaemic.

#### THE COMPOSITION OF THE PROTEINS OF EGGS FROM THE EXPERIMENTAL DIETS.

That the composition of the egg may be influenced by the diet of the fowl is clearly evidenced from the investigation of Bethke, Kennard and Sassaman [1927], who have shown that the vitamin A and vitamin D content of egg-yolk is largely, if not entirely, determined by the amount of these substances present in the diet. It is also well known that the calcium carbonate content of the egg shell may be varied greatly by different nutritional regimes.

It is to be presumed, however, that the amino-acid make-up of the proteins of the white and the yolk would be the same, irrespective of the composition of the proteins in the diet and that a diet containing inadequate proteins would result only in a decreased egg production and not also in a change in the composition of the egg-proteins.

In this connection, Pollard and Carr [1924] report remarkable variations in the composition of the total protein of eggs from pigeons fed on diets restricted to a single seed grain for protein and vitamins. While the total nitrogen of the egg was fairly constant the distribution of the nitrogen in the egg-protein varied greatly in eggs from different diets [see McCollum and Simmonds, 1928]. Of particular significance is their observation that only the eggs which gave a high melanin yield were hatchable. Gortner and Holm [1920] have shown that the formation of melanin is dependent upon the presence of tryptophan in the protein molecule. It is possible, then, that the protein moiety in the diet may have a profound influence on the composition of the proteins of the egg, thus influencing protein metabolism during embryonic development and incidentally also influencing incubational mortality.

Prange, Hauge and Carrick [1927] have attributed the nutritional failure of meat meal to a deficiency of its protein in tryptophan. Their experiments consisted in first supplementing the meat meal ration with gelatin, and, as no better growth was procured, it was indicated that meat meal was deficient in either cystine or tryptophan, or both. Tyrosine was adequately supplied by the corn and wheat of the basal ration. In order to make a further elimination, the meat meal ration was supplemented by caseinogen, which had been previously subjected to alkali treatment completely to remove cystine. From the improved growth which resulted they conclude that the amino-acid deficient in meat meal may be tryptophan. Sendju [1925] found that after three days of incubation, at the moment of the sudden appearance of blood pigment, there is a great reduction in the tryptophan content of the egg. After still longer incubation, during which the bile pigment appears, there is a further lowering of the tryptophan content, which suggests that in the hen's egg both free and bound tryptophan are significant in the synthesis of blood and bile pigment. Minot and Murphy [1926] suggest that animal protein may favour blood formation and conclude that adequate proteins, as well as iron, are necessary for the formation of haemoglobin. They also suggest that tryptophan may have a special ability to enhance blood formation.

It is suggested that a deficiency of tryptophan in meat meal and tankage may result in a similar deficiency in the eggs from hens on diets containing these products as the chief source of the protein. Assuming that the embryo, as is believed in the case of the adult animal, has not the power to synthesise the pyrrole nucleus required for the building up of haemoglobin, and that tryptophan is the normal source of this organic nucleus during incubation, then it is not impossible that a tryptophan deficiency may result in the development of an anaemic embryo weakened in vitality which ultimately dies.

An examination of the egg reveals that the iron is nearly all located in the yolk, where it exists in the form of an organic complex which is not fat-soluble and has certain protein-like properties. This substance was first studied by Bunge [1884] and named "haematogen," and was later studied in more detail by Hugounenq and Morel [1905]. They regard haematogen as a prosthetic group in the vitellin molecule and as being the precursor of the haemoglobin formed by the chick during the incubation of the egg. This theory is considerably strengthened by the fact that coincident with the great increase in the haemoglobin content of the chick on the 14th day of incubation as shown by Sendju [1927] there is a corresponding sudden decrease in the vitellin-phosphorus of the yolk as shown by Plimmer and Scott [1909]. Decomposition of haematogen by acids according to Hugounenq and Morel splits off a black, iron-containing pigment (2.60 % Fe) which they named "haematovin" because it represents in the hen's egg, "an incompletely differentiated and embryonic state of haematin." It seems probable that the synthesis of haemoglobin has been partly completed in the body of the hen before the egg is laid, the embryo completing the formation of haemoglobin from this "performed" compound. Anaemia in chick embryos may possibly be due to an inability to complete this synthesis or to a deficient supply of haematogen.

Copper according to Waddell *et al.* [1928; 1929, 1, 2] and Titus and Hughes [1928] must be considered another element necessary for haemoglobin synthesis, a deficiency of copper in the diet being a possible cause of nutritional anaemia. Drabkin and Waggoner [1929] on the other hand claim to have cured nutritional anaemia by means of a copper-free synthetic diet. It remains to be determined whether the copper content of the hen's egg is influenced by the copper content of the diet of the fowl, and whether nutritional anaemia in chick embryos from eggs deficient in copper may be possible.

#### *Separation of egg-proteins.*

The contents of the eggs obtained from the experimental birds in each of the pens described, *i.e.* Pens 28, 29, 30, 31 and 14, were separated into four protein fractions by the method of Plimmer and Rosedale [1925]. No attempt was made to separate the two proteins of the yolk, livetin and vitellin, the preparation being termed the egg-yolk-protein. The proteins of the white were separated into two fractions; one termed the egg-white-protein consisting of ovalbumin and ovoglobulin coagulated by boiling 0.4 % acetic acid, and the other the glycoprotein, ovomucoid, precipitated by absolute alcohol. To give sufficient material for analysis a composite of the egg-white-proteins from five eggs from each of seven birds in each pen was prepared. The same procedure was followed with the egg-yolk-proteins. In the case of the ovomucoid, where the yield per egg was small (0.1-0.3 g.), an amount insufficient for the purpose desired even with a 5-egg sample from an individual bird, composites of all the ovomucoid from each group were prepared. As we were particularly interested in the tankage group and as it was desired to obtain as many samples as

possible, although the number of eggs laid was limited, we prepared duplicate samples from the eggs of hen 1771 and triplicate samples from hen 1773.

As a control, the similar preparations of the proteins from five eggs, collected from five hens which were on range and which were receiving the poultry plant diet, were made and are designated the "normal."

The protein fractions separated were weighed, the average weight per egg in g. being as follows:

	Egg-white-protein	Egg-yolk-protein	Ovomucoid
Pen 28. Buttermilk powder	2.27	2.12	0.153
29. Fish meal	2.27	2.10	0.176
30. Tankage	2.11	2.14	0.155
31. Meat meal	2.38	2.19	0.195
14. Cod-liver meal	2.51	2.72	0.175

In the drying process on the glass plates, differences in the appearance of the yolks were observed. The yolks from any one hen, however, showed remarkable uniformity of appearance. Some would be transparent when nearly dry, others would show varying degrees of opacity. The depth of pigmentation was another variable property apparently uniform for an individual hen; and other such properties included size of yolk, and viscosity (whether "watery" or not so). It was noticed that the eggs of hen 535 on the cod-liver meal diet were large, and had very large yolks, which were light in colour and decidedly opaque, with a "watery" consistency. As a point of interest, the hatching record of this bird was consulted and it was found that in the month previous, namely April, 22 chicks had hatched from 23 eggs.

In the precipitation of the egg-white-protein with 0.4 % acetic acid it was found important to have the acid boiling briskly, to add the white slowly and with stirring, and to boil rapidly for at least three minutes. Even with these precautions poor separations were sometimes obtained, in which case repeated filtration and boiling were necessary. In the case of the hen 1787 this difficulty was unusually marked. Half an hour's boiling failed to bring about coagulation, nor was washing by decantation successful, a gelatinous mass being formed throughout the liquid. The separation was finally made by repeated washing and centrifuging, resulting in considerable loss. The hatching record of this hen revealed exclusively dead germs and infertile eggs.

A yellowish tinge in certain egg-whites was noticed to pass through with the ovomucoid fraction, being separated from this during the extraction with absolute alcohol.

#### *Analyses of the protein samples.* (Tables II, III and VII.)

The following determinations were made in duplicate.

(1) *Total nitrogen* (Kjeldahl).

(2) *Loss of weight on drying.* The samples as prepared contained a considerable amount of volatile matter in the form of alcohol and water. Weighed portions of about 0.5 g. were heated in the vacuum oven at 90°/5 mm. for six



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hours, and the process repeated until two consecutive weighings differed by less than 0.5 mg.

Table II. *Analysis of egg-white-proteins.*

Sample	% loss in weight on heating in vacuum at 90°	Nitrogen (% dry weight)	Total amino-N in tryptic digest (% total N)
Normal	9.93	15.09	54.4
Pen 28. Buttermilk powder			
1751	8.06	15.13	55.0
1752	9.33	15.16	48.8
1753	9.01	14.90	51.2
1754	9.43	15.30	53.6
1755	10.86	15.51	52.4
1756	8.47	15.38	50.5
1758	9.07	15.05	50.9
Pen 29. Fish meal			
1762	9.18	14.89	53.1
1763	9.17	15.18	48.7
1764	10.73	15.60	48.4
1765	10.22	14.99	49.4
1766	9.28	15.38	48.1
1767	11.68	15.58	52.6
1768	10.95	15.10	51.1
Pen 30. Tankage			
1119	9.38	15.28	56.4
1771 (1)	10.25	15.55	53.9
1771 (2)	10.10	15.29	55.8
1773 (1)	12.68	14.91	53.2
1773 (2)	9.40	15.20	55.5
1773 (3)	8.64	15.50	55.1
1775	13.32	14.85	48.7
1776	9.50	14.76	53.5
1777	9.63	15.15	52.8
Pen 31. Meat meal			
1783	11.96	15.31	50.0
1784	9.95	15.15	52.1
1785	9.18	15.15	47.0
1786	10.42	15.14	47.0
1787	10.14	15.30	48.3
1789	9.96	15.15	49.6
1790	16.02	14.77	44.5
Pen 14. Cod-liver meal			
527	10.57	14.90	55.4
529	8.00	15.32	55.0
531	13.55	15.15	50.0
533	11.29	15.28	51.9
535	8.88	15.21	52.6
1012	10.70	15.19	48.6
1014	9.17	15.17	52.8

(3) *Total ash.* The ash content of the egg-white-proteins was so low (0.48 % in the case of the egg-white-protein from Pen 28) that it was not considered significant. The same applies to the ovomucoid samples. In the case of the egg-yolk-protein the ash content of the individual samples, calculated as % of the dry weight of protein, is included in Table III.

Table III. *Analysis of egg-yolk-proteins.*

Sample	% loss in weight on vacuum drying at 90°	Nitrogen (% dry weight)	Ash (% dry weight)	Total amino-N in tryptic digest (% total N)
Normal	8.22	14.39	4.73	43.1
Pen 28. Buttermilk powder				
1751	7.78	14.00	4.77	52.6
1752	7.90	14.38	5.16	50.1
1753	7.67	14.29	4.92	47.4
1754	7.37	14.30	5.19	47.2
1755	7.32	14.09	5.14	48.1
1756	7.91	14.48	4.87	37.6
1758	8.49	14.30	5.34	48.6
Pen 29. Fish meal				
1762	7.48	14.41	5.01	45.6
1763	8.09	13.95	4.52	39.4
1764	7.42	12.95	4.84	49.6
1765	8.18	14.11	4.67	47.7
1766	7.55	14.20	4.98	44.1
1767	8.15	14.35	5.06	43.5
1768	9.37	14.15	5.33	45.1
Pen 30. Tankage				
1119	7.58	14.60	4.90	47.2
1771 (1)	7.77	14.35	4.87	48.3
1771 (2)	7.65	14.20	4.84	50.7
1773 (1)	8.32	14.46	5.03	52.0
1773 (2)	8.78	14.65	5.02	54.1
1773 (3)	8.05	14.73	4.98	49.3
1775	8.72	14.66	4.83	50.8
1776	8.20	14.68	4.49	53.2
1777	8.23	14.69	5.06	54.1
Pen 31. Meat meal				
1783	7.70	14.35	5.03	50.5
1784	8.45	14.34	5.09	49.1
1785	8.28	14.32	5.06	45.2
1786	7.97	14.08	4.96	49.4
1787	7.75	14.21	4.94	46.1
1789	9.53	14.41	5.19	45.8
1790	8.24	14.39	4.97	45.5
Pen 14. Cod-liver meal				
527	7.65	14.46	4.79	51.0
529	6.72	14.55	4.70	51.8
531	6.78	14.61	4.94	57.5
533	8.64	14.50	5.18	52.4
535	8.25	14.69	4.69	48.7
1012	7.43	14.31	4.84	53.8
1014	8.19	14.55	4.71	48.0

(4) *Amino-acids.* The tyrosine, tryptophan and cystine content of these egg-proteins was determined. In view of the large number of determinations to be made it was considered that only enzymic hydrolysis of these proteins could be used and that the subsequent determinations of these amino-acids in the hydrolysates would have to be made by colorimetric methods. The following procedure was adopted.

Weighed quantities of the protein samples equivalent to 0.5 g. protein ( $N \times 6.25$ ) were transferred to 125 cc. Erlenmeyer flasks. A trypsin solution

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containing 1.0 g. "Trypsin<sup>1</sup>" in 100 cc. of 0.4 % Na<sub>2</sub>CO<sub>3</sub> was prepared. 25 cc. of this trypsin solution + 1 cc. toluene were incubated in tightly stoppered flasks for 10 days at 37.5°. During the first 24 hours the flasks were periodically gently shaken until the protein had gone into solution. At the completion of the digestion, the hydrolysates were acidified by adding 2 cc. 14N H<sub>2</sub>SO<sub>4</sub> giving a total volume of 28 cc. in N/1 H<sub>2</sub>SO<sub>4</sub>. On adding 2 cc. 14N H<sub>2</sub>SO<sub>4</sub> to the tryptic digests of the egg-yolk-proteins a heavy brown precipitate formed. This substance was believed to be haematovin (p. 1617). The acidified digests were centrifuged and the clear supernatant liquids decanted. That the tyrosine or tryptophan content of the digest had not been diminished by removing this precipitate was shown by the fact that the precipitate washed with N H<sub>2</sub>SO<sub>4</sub> did not give the Millon or Hopkins-Cole reaction. The flasks were then placed in the refrigerator until required. Before commencing the amino-acid determinations the digests were shaken with a small amount of kaolin and filtered clear. Total amino-nitrogen was determined on 5 cc. of the hydrolysate using Van Slyke's macro-apparatus.

### *Tryptophan.*

Tryptophan determinations were first made on 2 cc. of the tryptic digests of the egg-white-proteins and egg-yolk-proteins by the method of Ragins [1928]. Repeated determinations of the tryptophan content of the same sample of egg-white- or egg-yolk-protein by this method gave results which agreed very closely. It was found, however, that the tryptophan values of each seven egg-white- or egg-yolk-protein preparations from eggs from the same pen on the same diet were not constant, as we had anticipated, but fluctuated quite considerably. As a check tryptophan determinations were made on 2 cc. of the tryptic digests of the egg-white-proteins using the phenol reagent of Folin and Marenzi [1929, 1]. Direct determinations were also made on 1 g. samples of the egg-white-proteins by the method of May and Rose [1922] as modified by Boyd [1929].

Again variations were found in the tryptophan content of the seven egg-white-protein preparations of eggs from the same pen on the same diet, which were much greater than the experimental error of duplicate determinations on the same sample. Of particular significance, however, was the fact that there was no relationship between the fluctuations in the tryptophan content of the egg-white-proteins as determined by the three colorimetric methods, suggesting that the major factor contributing to these variations was the final colorimetric method of determination.

Table IV shows the results of these tryptophan determinations. Each value represents the mean tryptophan content of all the samples of egg-white- or egg-yolk-protein from each pen as enumerated in Table II. The probable error of the mean as determined by Peter's formula  $\left( \text{P.E.} = 0.8453 \cdot \frac{\sum V}{n \sqrt{(n-1)}} \right)$  is also given.

<sup>1</sup> Digestive Ferments Co., Detroit, Mich., U.S.A.

Table IV. *Estimation of tryptophan in egg-white- and egg-yolk-proteins.*

Sample		Results expressed as % of dry weight of protein.			Egg-yolk-protein. By Rugins's reagent
		Egg-white-protein			
		By Rugins's reagent	By Folin's reagent	By May and Rose method	
Pen 28.	Buttermilk powder	1.37 ± 0.019	1.27 ± 0.018	1.53 ± 0.048	1.49 ± 0.019
Pen 29.	Fish meal	1.31 ± 0.028	1.20 ± 0.011	1.53 ± 0.020	1.47 ± 0.016
Pen 30.	Tankage	1.40 ± 0.025	1.32 ± 0.029	1.71 ± 0.043	1.54 ± 0.015
Pen 31.	Meat meal	1.25 ± 0.014	1.16 ± 0.018	1.58 ± 0.022	1.48 ± 0.013
Pen 14.	Cod-liver meal	1.51 ± 0.015	1.20 ± 0.040	1.76 ± 0.014	1.53 ± 0.023

Compared with the other methods the May and Rose determination was not so accurate. A few values had such a great variation from the mean value that it was considered best to discard these, using Wright's criterion of rejection, *i.e.* discarding all values showing variations from the mean greater than five times the probable error.

Because of the variability in the results of the tryptophan determinations on these egg-proteins from eggs from different hens on the same diet it was felt that, to detect slight variations in the amino-acid content of eggs from hens on different diets, more accurate methods of analysis would require to be evolved than the colorimetric methods which we purposed using. However, having found that changing the source of protein in the diet of the hen had a marked influence on the mortality of the embryo during incubation, our objective was to determine whether this effect was accompanied by gross changes in the composition of the egg-protein so far as the tyrosine, tryptophan or cystine content was concerned. It was concluded (see discussion) that the limits of accuracy of the colorimetric methods of analysis which we used would permit the drawing of definite conclusions in this regard.

The tryptophan content of the tryptic digests of the egg-yolk-protein and ovomucoids could only be determined by using Rugins's vanillin-HCl reagent. The colour developed with the phenol reagent was a different shade of blue from that developed by standard solutions of tryptophan or tyrosine. The colour developed by caseinogen, used as a standard, with *p*-dimethylamino-benzaldehyde was not comparable with the different shade of blue developed by the egg-yolk-protein or ovomucoid. The results of the tryptophan determinations on the ovomucoids is given in Table VII.

#### *Tyrosine.*

Tyrosine determinations were made on the tryptic digests by the method of Folin and Marenzi [1929, 1]. In determining the tyrosine content of the egg-white-proteins and the ovomucoids it was found that invariably in the unknown solutions after heating with HgSO<sub>4</sub> and cooling a white cloudy precipitate formed, which made the subsequent colorimeter reading impossible. To overcome this difficulty a slight modification of the procedure was made. After centrifuging the HgSO<sub>4</sub> precipitate containing the tryptophan, the tyrosine-containing supernatant liquid was transferred to a 50 cc. conical

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centrifuge tube instead of a 100 cc. volumetric flask. After heating the unknown solution with  $\text{HgSO}_4$  and cooling, a white cloudy precipitate formed. This precipitate was centrifuged and the supernatant liquid transferred to a 100 cc. volumetric flask. The precipitate was then washed with 10 cc.  $N \text{H}_2\text{SO}_4$ , centrifuged and the wash liquid added to the main solution. The remainder of the procedure was carried out in the usual manner, perfectly clear colorimetric readings being now obtained. The substance removed in the precipitate did not give any colour with Millon's reagent.

Variations in the tyrosine content of individual egg-protein samples from eggs from hens on the same diet were found as in the results of the tryptophan determinations. The mean tyrosine content of the egg-yolk- and egg-white-proteins from each diet and the probable error of the mean is given in Table V.

Table V. *Estimation of tyrosine in egg-white- and egg-yolk-proteins.*

Results expressed as % of dry weight of protein.		
Sample	Egg-white-protein	Egg-yolk-protein
Pen 28. Buttermilk powder	$4.96 \pm 0.037$	$4.85 \pm 0.054$
Pen 29. Fish meal	$5.02 \pm 0.029$	$5.06 \pm 0.029$
Pen 30. Tankage	$4.97 \pm 0.046$	$5.25 \pm 0.038$
Pen 31. Meat meal	$4.93 \pm 0.049$	$5.13 \pm 0.028$
Pen 14. Cod-liver meal	$5.14 \pm 0.055$	$5.26 \pm 0.014$

The results of the tyrosine determinations on the ovomucoids are given in Table VII.

### *Cystine.*

Recently Folin and Marenzi [1929, 3] have described a method for the preparation of the uric acid reagent completely free from the phenol reagent. The uric acid reagent was prepared according to the directions and was found to give no blue colour with the standard tryptophan solution. We could see no objection against determining the cystine content of these egg proteins by the use of this purified uric acid reagent with the tryptic digests. Glutathione, the only other substance which might have made the determination inaccurate, is not present in the unincubated egg. This procedure, of course, involves the assumption either that cystine is liberated quantitatively by tryptic digestion of these proteins or that comparatively the tryptic digestion (cystine liberated) of the different samples of the same egg-protein has proceeded to the same extent. Cystine determinations were made on a 5 cc. aliquot of the decolorised tryptic digests by the method of Folin and Marenzi [1929, 2]. In Table VI is

Table VI. *Estimation of cystine in egg-white- and egg-yolk-proteins.*

Results expressed as % of dry weight of protein.		
Sample	Egg-white-protein	Egg-yolk-protein
Pen 28. Buttermilk powder	$2.14 \pm 0.050$	$1.70 \pm 0.045$
Pen 29. Fish meal	$2.09 \pm 0.046$	$1.70 \pm 0.036$
Pen 30. Tankage	$2.22 \pm 0.046$	$1.66 \pm 0.041$
Pen 31. Meat meal	$1.98 \pm 0.028$	$1.72 \pm 0.020$
Pen 14. Cod-liver meal	$2.20 \pm 0.042$	$1.80 \pm 0.016$

shown the average cystine content of the egg-white- and egg-yolk-proteins from each diet and the probable error of the average value.

The results of the cystine determinations on the ovomucoids are given in Table VII.

Table VII. *Analysis of ovomucoids.*

Sample	% loss in weight on drying <i>in vacuo</i> at 90°	Total nitrogen (% of dry weight)	Determinations on tryptic digest				
			Total amino-N (% of total N)	Tyrosine*	Tryptophan*	Cystine*	Total sulphur
Pen 28. Buttermilk powder	11.47	13.50	44.4	4.00	2.33	6.35	2.39
Pen 29. Fish meal	10.39	13.55	45.0	4.26	2.34	5.94	2.27
Pen 30. Tankage	10.36	13.48	45.7	3.99	2.09	6.36	2.24
Pen 31. Meat meal	10.08	13.51	45.9	4.22	2.36	5.82	2.24
Pen 14. Cod-liver meal	11.07	13.20	44.6	4.32	1.95	6.49	2.20

\* Results expressed as % of the total protein (N × 6.25).

As a check on the fluctuations in the cystine content of these proteins, which is particularly marked in the case of the ovomucoids, total sulphur determinations were made on the ovomucoid by the sodium peroxide method [Assoc. of Official Agric. Chemists, 1924]. The disparity in the cystine content was not correlated with the total sulphur content as shown in Table VI. Cystine determinations have been made on the "normal" egg-white- and egg-yolk-protein and on a sample of purified caseinogen by H<sub>2</sub>SO<sub>4</sub> hydrolysis and compared with the results obtained by tryptic digestion.

	Cystine	
	H <sub>2</sub> SO <sub>4</sub> hydrolysate	Trypsin hydrolysate
Caseinogen	0.30	0.38
Normal albumin	1.77	2.12
„ yolk	1.56	1.88

In each case the cystine content obtained by the same colorimetric procedure after H<sub>2</sub>SO<sub>4</sub> hydrolysis was lower than that obtained by tryptic digestion. Plimmer and Lowndes [1927] have shown that cystine is changed by boiling with acids, losing 7 % of its nitrogen. The cystine content of ovomucoid is very high compared with that of the other egg-proteins. The method of expressing these results in Table VII makes the figure appear high. The corresponding results expressed as % of the original dry weight of material were 5.45, 5.04, 5.50, 5.00 and 5.60 respectively.

#### *Determination of the iron content of the egg-yolk-proteins.*

In determining the iron content of the egg-yolk-protein preparations we hoped to obtain an indication of any variation in the content of iron present in protein combination, a low iron content being a criterion of a low supply of this precursor of haemoglobin.

Iron determinations were made by a slight modification of the method of

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Kennedy [1927]. As we desired figures for the ash content of our samples, the digestion method of Kennedy was not used. Instead, duplicate samples of about 0.5 g. of the egg-yolk-protein were ashed in platinum dishes at 500°-600° for at least 10 hours. The ash was weighed, then moistened with dilute HNO<sub>3</sub> and evaporated to dryness and again heated for a few minutes in the muffle, to ensure complete oxidation of all the iron. The cooled residue was then dissolved in 5 cc. of dilute HCl (1 : 1). The solution was then made up to 50 cc. and 10 cc. were taken for the iron estimation. The remainder of the determination was made by the thiocyanate method of Kennedy, 10 cc. of a solution containing 0.005 mg. Fe per cc. being used as the standard.

Table VIII. *Results of iron determinations on egg-yolk-protein.*

Sample	% iron in yolk-protein (dry weight)		% iron in yolk-protein (N × 6.25)	% iron in yolk-protein ash
	(1)	(2)		
Normal	0.037	0.038	0.042	0.80
Pen 28. Buttermilk powder				
1751	0.057	0.064	0.068	1.28
1752	0.044	0.048	0.051	0.89
1753	0.057	0.055	0.063	1.14
1754	0.055	0.061	0.065	1.12
1755	0.057	0.060	0.066	1.15
1756	0.045	0.046	0.050	0.94
1758	0.049	0.046	0.053	0.90
Pen 29. Fish meal				
1762	0.050	0.055	0.059	1.06
1763	0.043	0.042	0.049	0.92
1764	0.041	0.041	0.051	0.85
1765	0.041	0.040	0.046	0.87
1766	0.034	0.033	0.038	0.67
1767	0.041	0.042	0.046	0.83
1768	0.044	0.044	0.050	0.84
Pen 30. Tankage				
1119	0.032	0.032	0.032	0.67
1771 (1)	0.039	0.032	0.035	0.78
1771 (2)	0.052	0.051	0.051	1.08
1773 (1)	0.052	0.049	0.050	1.01
1773 (2)	0.055	0.056	0.055	1.10
1773 (3)	0.056	0.057	0.056	1.14
1775	0.053	0.051	0.052	1.08
1776	0.050	0.049	0.050	1.11
1777	0.053	0.052	0.052	1.05
Pen 31. Meat meal				
1783	0.037	0.037	0.041	0.73
1784	0.043	0.044	0.049	0.86
1785	0.040	0.038	0.044	0.77
1786	0.040	0.040	0.045	0.82
1787	0.039	0.041	0.044	0.86
1789	0.029	0.031	0.033	0.57
1790	0.056	0.057	0.063	1.15
Pen 14. Cod-liver meal				
527	0.042	0.043	0.047	0.88
529	0.039	0.039	0.043	0.83
531	0.042	0.045	0.048	0.88
533	0.040	0.039	0.044	0.76
535	0.028	0.029	0.031	0.62
1012	0.048	0.049	0.054	1.01
1014	0.046	0.045	0.050	0.97

The results are shown in Table VIII. The iron contents of the diets fed was also determined and were as follows:

				% Fe in the diet
Pen 28.	Buttermilk powder	...	...	0.0053
Pen 29.	Fish meal	...	...	0.0068
Pen 30.	Tankage	...	...	0.0155
Pen 31.	Meat meal	...	...	0.0095

*Determination of the copper content of eggs from different diets.*

Owing to the relatively small proportion of copper in biological materials it was necessary to find a method which would bring the amount of copper contained in one egg well within the range of sensitivity of the method chosen.

The copper content of egg-yolk is given as 20 mg. per kg. of dry material by Fleurent and Levi [1920], as 2.5 mg. per kg. by McHargue [1925] and as 8.0 mg. per kg. by Lindow, Elvehjem and Peterson [1929] and as 1.8 mg. per kg. of fresh material (equivalent to 3.6 mg. per kg. of dry material) by Guerithault [1927]. The last mentioned author cites egg-white as being free from copper. Taking the last fact into consideration, and taking the weight of the yolk and white of an average egg as 45 g. of fresh material, 8 g. of which is yolk dry matter containing virtually all the copper, a yield of copper per egg of from 0.16 mg. to 0.02 mg. was anticipated.

For the estimation it was decided to try the method of Biazzo [1926] which has been used extensively by Elvehjem and Lindow [1929]. Unfortunately the high iron content of eggs was found to cause a discoloration of the chloroform layer making it difficult to compare with the standard. Elvehjem and Lindow give a somewhat unsatisfactory method for removing iron by the use of copper-free zinc. Instead of this the ash solution was treated with an excess of  $\text{NH}_4\text{OH}$ ; the supernatant liquid then contains all the copper, free from iron. The procedure of Elvehjem and Lindow modified in this way was used and proved that a good recovery of copper from iron and phosphate mixture could be obtained.

Copper determinations were made on eggs from the birds in Pens 28, 29, 30 and 31. The eggs were weighed, boiled hard, peeled and the shell with its membranes discarded. The edible portion was ashed in platinum, dissolved in 1:1 HCl, excess of strong  $\text{NH}_4\text{OH}$  added, centrifuged to separate the gelatinous precipitate, the washings poured off and the precipitate redissolved in HCl and reprecipitated with  $\text{NH}_4\text{OH}$ , centrifuged and the supernatant liquid combined with the first ammoniacal solution. The process was repeated to ensure the recovery of all the copper from the gelatinous precipitate insoluble in the ammonium hydroxide. The combined ammoniacal washings were evaporated to a volume of about 5 cc. and washed into a 50 cc. stoppered cylinder. The determinations were completed by the method of Elvehjem and Lindow.

The results are given in Table IX. No attempt has been made to express averages. The reason for this is the great variation in copper content observed



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in different eggs from the same hen on the same ration. For example, three eggs from hen 1789 gave a copper content of 0.04 mg., 0.09 mg., and 0.14 mg. respectively.

Table IX. *Results of copper determinations on eggs from different diets.*

Sample	Wt. of edible part of egg (g.)	% of ash in edible part	Total Cu (mg.)	Cu in edible part (mg./kg.)
Pen 28. Buttermilk powder				
1754	40.4	0.96	0.15	3.7
1754	45.1	0.92	0.05	1.1
1755	48.3	0.97	0.05	1.0
1758	40.5	1.02	0.10	2.5
1758	42.1	0.98	0.06	1.4
1758	44.5	1.00	0.05	1.1
1758	44.5	0.93	0.09	2.0
1759	52.4	0.86	0.11	2.1
Pen 29. Fish meal				
1762	43.5	0.98	0.07	1.6
1763	52.2	—	0.03	0.6
1763	52.0	0.99	0.05	1.0
1764	45.7	1.01	0.04	0.9
1765	46.7	0.88	0.11	2.4
1766	46.9	0.96	0.12	2.6
1766	50.3	0.91	0.06	1.2
1766	49.5	0.88	0.13	2.6
1768	46.2	0.88	0.05	1.1
1768	46.7	0.85	0.07	1.5
1768	46.4	0.86	0.09	2.0
Pen 30. Tankage				
1101	42.7	0.99	0.07	1.6
1101	42.6	0.98	0.05	1.2
1101	42.1	0.81	0.08	1.9
1101	37.5	1.08	0.08	2.1
1101	42.5	0.92	0.07	1.6
1771	42.0	0.97	0.14	3.3
1771	43.1	0.94	0.08	1.9
1771	42.1	0.91	0.08	1.9
1771	41.3	0.96	0.14	3.4
1776	42.3	0.94	0.08	1.9
1776	39.7	0.99	0.08	2.0
1776	40.3	0.96	0.17	4.2
1776	43.2	0.95	0.08	1.8
1777	44.3	0.95	0.15	3.4
1778	40.8	0.96	0.05	1.2
Pen 31. Meat meal				
1783	48.5	0.90	0.05	1.0
1783	46.8	1.05	0.08	1.7
1784	42.7	0.87	0.07	1.6
1784	52.4	0.86	0.09	1.7
1785	51.5	0.93	0.19	3.7
1785	43.8	0.92	0.06	1.4
1785	44.8	0.91	0.14	3.1
1786	39.7	1.13	0.04	1.0
1789	44.8	0.95	0.09	2.0
1789	38.8	0.97	0.14	3.6
1789	46.3	0.96	0.04	0.9

The copper content of the protein supplements and basal ration used in these experiments was also determined with the following results:

	Copper content (mg./kg.)
Buttermilk powder	3.8
Fish meal	4.2
Tankage	7.2
Meat meal	5.4
Cod-liver meal	13.35
Basal mash	3.4

## DISCUSSION.

There is no great difference in the weights of the different protein fractions from eggs in Pens 28, 29, 30 and 31. In the cod-liver meal group, Pen 14, the eggs gave a greater yield of egg-white- and egg-yolk-protein, particularly the latter. The egg-white- and egg-yolk-protein weights were very constant in the eggs from the different diets. There is no appreciable difference in the weights of the ovomucoid obtained from the different groups.

The total nitrogen content of the egg-white-, egg-yolk- and ovomucoid-protein in the different groups was fairly constant although discrepancies occurred which were much greater than could be accounted for. In the case of hen 1764 the nitrogen content of the egg-yolk-protein was much below the average, this figure having been confirmed by triplicate determinations. The total nitrogen content of the cod-liver meal ovomucoids was also slightly below the average, the nitrogen content of the other ovomucoid preparations being very constant. The ash content of the egg-yolk-proteins, Table III, as was the case with the total nitrogen, was fairly constant but varied to a greater extent than could be explained on the basis of experimental error, as duplicate determinations gave very close results. There was no correlation between the total nitrogen and the ash content, indicating that the slight variations were not due to differences in the purity of the preparations.

The tryptic digests of these proteins, the results of the analysis of which is given in Tables IV, V and VI, were all made with an aliquot of the same trypsin solution under identical conditions of temperature and time. The fluctuations in the total amino-nitrogen in the tryptic digests of the egg-white- and egg-yolk-protein (Tables II and III) are noteworthy. Duplicate determinations in no case varied more than 0.5%. The total amino-nitrogen liberated by tryptic digestion of the ovomucoids was very constant.

The egg-white-protein of eggs of poor hatchability, *e.g.* from Pen 30, contained just as much tryptophan (Table IV) as the egg-white-protein of eggs of high hatchability, *e.g.* those of Pen 14. The variation with diet in the tryptophan content of the egg-white-protein as determined by the three methods is within the experimental error of the methods of analysis. No significant difference in the tryptophan content of the egg-yolk-protein from different diets (Table IV) was found by Ragins's method. Changing the tyrosine and tryptophan content of the diet of the hen by varying the source of protein did not result in changes of the same order in the content of these amino-acids in the egg-proteins. The variations in the tyrosine, tryptophan and cystine con-

tent of different samples of egg-white- and egg-yolk-protein from the same diet, as the result of sampling or inaccuracies in the methods of analysis, were just as great as the difference between different diets.

Of particular importance so far as this paper is concerned was the fact that there was no clear evidence that the diet of the hen had any influence on these values, and that the proteins from eggs of poor hatchability were found to contain just as much tyrosine, tryptophan and cystine as the proteins of eggs of high hatchability. It has to be admitted, however, that any slight changes in the amino-acid content of the egg-proteins could not be detected in these estimations and may possibly occur. The tyrosine, tryptophan and cystine content of the "normal" samples of egg-white- and egg-yolk-protein expressed as % of dry weight of protein was as follows:

	Egg-white-protein	Egg-yolk-protein
Tyrosine	5.22	4.82
Tryptophan by Ragins's reagent	1.41	1.48
"    by Folin's reagent	1.30	—
"    by May and Rose method	1.62	—
Cystine	2.12	1.88

There was no relationship between the variations in the tyrosine, tryptophan or cystine content of the ovomucoid (Table VII) from eggs from the different diets and the hatchability of these eggs. Taking into account the fact that only a single sample of ovomucoid from each diet was analysed, and the fluctuations previously found in the amino-acid content of the egg-white- or egg-yolk-protein from the same diet, the variations recorded in Table VII cannot be regarded as significant.

It will be noted that there is a difference in the tryptophan content of the egg-white-protein with different methods of determination. The method of May and Rose gave the highest tryptophan content while the results with the Folin and Marenzi procedure are uniformly lower than those obtained by the vanillin-HCl reagent of Ragins.

The great variation in the copper content, as shown in Table IX of eggs from the same hen on the same ration is noteworthy. The comparatively high copper content of buttermilk powder is of interest in view of the fact that the copper content of cow's milk is reported as 0.15 mg. per litre by Elvehjem, Steenbock and Hart [1929]. The contact with copper during the processing may raise the copper content of buttermilk powder within widely varying limits. The high copper content of cod-liver meal is of interest in view of the high copper content of mammalian livers. The copper content of tankage is quite considerable, probably because of the blood-meal which is incorporated in it.

Considerable variation in the iron content of the egg-yolk-proteins is shown in Table VIII. Variations from 0.031 % iron in the case of 535 to 0.066 % iron in the case of 1755, were obtained. Since the duplicates agree fairly closely these variations can be regarded as quite significant. There is also

a slight tendency for the iron content of the egg-yolk-protein from the eggs in the buttermilk powder group to be the highest of all. However, although the variations in iron content are considerable, there is no pronounced trend in the results which could be attributed to the influence of diet. The iron content of tankage is considerably greater than that of the other protein supplements, which is no doubt the result of the inclusion of blood-meal.

Compared with other feeds the protein supplements employed are liberally supplied with iron and copper, while the basal mash itself contains very considerable amounts of iron (0.0061 % Fe) and copper (3.4 mg. Cu per kg.). The possibility of nutritional anaemia in poultry as the result of a deficiency of iron or copper in the diet would seem to be extremely remote.

#### SUMMARY.

(1) The source of protein in the diet of the hen was found to have a marked influence on the mortality of the embryos during incubation.

(2) No significant difference was found, however, in the composition of the proteins of eggs of poor hatchability so far as the total nitrogen, total amino-nitrogen, tyrosine, tryptophan and cystine content was concerned and the composition of the proteins of eggs of high hatchability. There was no clear evidence that the diet of the hen had any influence on these values.

(3) Total ash and iron determinations on the egg-yolk-proteins showed variations greater than could be accounted for by the limitations in the analytical methods used. There was little or no evidence, however, that the total ash and iron content of the egg-yolk-proteins was influenced by diet.

(4) The copper content of the hen's egg was found to be an extremely variable quantity.

(5) The results of these analyses of eggs from hens on different diets so far offer no explanation of the differences in their hatchability, nor do they throw any light on the cause of the anaemic state which has been found to be one of the major conditions associated with the mortality of embryos from tankage and meat meal eggs during the early stages of incubation.

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