STUDIES ON THE UTILIZATION OF VITAMIN A IN THE DEVELOPING CHICK EMBRYO

by

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INTRODUCTION AND REVIEW OF LITERATURE

The study of the embryonic utilization of nutrients is an old and important subject relating to the bearing or hatching of strong, healthy offspring, which is important both from an economic and a humanitarian viewpoint. The viability of the offspring is dependent to a great extent upon the proper nutrition of the embryo.

Laboratory experimentation on the utilization of various nutrients by the embryo usually has been carried on using the fertile avian egg, which is peculiarly adapted for these studies since the embryo can be removed easily from the remaining yolk material. Needham (1) has written an excellent review of the information available on this subject up to 1931.

One of the earliest investigations of this problem was a study of the weight changes of the embryo during its development. In 1857, Falck (2) reported the embryonic weight changes of the chick embryo. His data indicated a constantly increasing rate of weight gain during the embryonic growth. The data of Falck (2) are in substantial agreement with those from other workers (cited by Needham, 3). The more recent and extensive work of Penquite (4) indicated that the data approached a straight line when the logarithm of dry weight, wet weight, or total nitrogen content of the chick embryo was plotted against the days of incubation, i.e., embryonic age.

In addition to studies of weight changes, many investigations on the variation in content of specific nutrients in the embryo

have been made. Needham (5) estimated the total carbohydrate content of the chick embryo during various stages of development. The carbohydrate content exhibited a constantly increasing rate of transfer to the embryo as its age increased. Sendju (6) determined the content of the amino acids cystine, lysine, arginine, histidine, tyrosine, and tryptophane in the chick embryo. These substances were found to increase in amount with increase in the embryonic age in a manner similar to that of carbohydrate content. In 1927, Needham (7), using Rose's modification of the Kjeldahl method, found that the non-protein nitrogen content of the chick embryo increased in a similar exponential manner. Murray (8), among others, has reported the fatty acid content of the embryo at various stages of development. His data also indicated this same constantly increasing trend in the transfer curve of the latter nutrient.

Metabolism of inorganic substances by the avian embryo has been studied by Iseki (9). He found that by plotting data for potassium, sodium, chloride and sulphate ion content, the same constantly increasing transfer curve was obtained. Plimmer and Lowndes (10) found the transfer of calcium to the chick embryo followed the same type of curve as the aforementioned ions.

A search of the literature revealed only scant information on the transfer of the various vitamins to the embryo during different stages of development. In 1949, Scrimshaw et al. (11) reported that the concentration of thiamine in the chick embryo increases during incubation in an exponential fashion. Barnett

and Bourne (12) indicated the distribution of ascorbic acid in the embryo during the first four days of incubation. Taylor et al. (13) reported the concentrations of eight of the B-complex vitamins in the liver, brain, and hearts of 12-day old embryos and of day old chicks. Holmes (14) investigated the content of vitamin A in livers and unabsorbed yolk sacs of 18-day embryos, and of 6 and 24 hour chicks. Suomalainen (15) reported that the chick embryo used 350 I.U. of vitamin A during the last two weeks of incubation; while Lissot and Caridroit (16) reported that 350 I.U. of vitamin A per egg were necessary for complete development and hatching of the chick.

The importance of vitamin A not only in poultry nutrition but in nutrition of other animals as well has been established. The first published reports on vitamin A appear to be those of McCollum and Davis (17) in 1913, and of Osborne and Mendel in 1913, (18) and 1914, (19). Their work indicated the necessity of a fat-soluble factor, which was found in egg yolk for the normal growth of rats. Detection of vitamin A in egg yolk by rat assay was reported by Murphy and Jones (20) in 1924, and by Bethke et al. (21) in 1927. The Carr-Price (22) reaction for the estimation of vitamin A content was applied to egg yolk in 1932 by Euler and Klussman (23). The following year the latter workers (24) reported that the normal hen's egg contained the equivalent of 20 U.S.P. units of vitamin A per gram of yolk. In 1940, Sjollema and Donath (25) found that the normal hen's egg contained from 200 to 300 I.U. of vitamin A; their values were based on a

colorimetric determination. Gillam and Heilbron (26) in 1935, published a method for the chromatographic separation of the various carotenoid pigments and vitamin A from egg yolk.

Schroeder et al. (27) reported on the necessity of vitamin A in poultry rations for the maintenance of adequate growth. The mortality of chicks during incubation is related to the quantity of vitamin A in the yolk, Zaborowski (28). Thomas and Quackenbush (29) showed that the vitamin A content of the yolk is dependent upon the amount of vitamin A in the feed and that the efficiency of the hen in transferring this vitamin to the yolk decreases with increasing vitamin A intake. Sherwood and Fraps (30), Bethke et al. (21), and Payne and Hughes (31) have shown that increasing the quantity of vitamin A in the feed increases the hatchability of eggs.

In view of the importance of vitamin A in poultry nutrition and the dearth of information on the embryonic utilization of this vitamin, further investigation seemed desirable. This information is essential in the formulation of a general theory concerning the role of vitamin A during the fetal life of an animal. Therefore, the purpose of the present investigation was to determine the variations of content and state of vitamin A of eggs and of chick embryos during various stages of development.

VARIATIONS IN THE VITAMIN A CONTENT OF THE CHICK EMBRYO AND ITS RESIDUAL YOLK SAC, AND IN INFERTILE EGGS

Procedure

Management of the experimental fowls. The eggs that were used for determination of the variations in the vitamin A content of the chick embryo during its development were collected from a group of White Leghorn hens. The hens were maintained on a pigment-low ration, as shown below, since Thompson et al. (32) reported that the presence of a high carotenoid concentration in the egg yolk interfered with the determination of vitamin A by the Carr-Price reaction (22), which was selected to be used as the method in the present study.

The composition of the pigment-low ration was as follows:

Corn, white, ground	120 pounds
Wheat shorts (16 percent)	20 "
Oats, ground	20 "
Bran, wheat	10 "
Meat and Bone scrap (50 percent)	7 "
Soybean oil meal (42 percent)	9 "
Fish Meal (65 percent)	3 "
Brewer's yeast	3 "
Dried skim milk	3 "
Calcium carbonate	2 "
Sodium chloride	1 "
Fish solubles	4 "
Vitamin D (D-Sec)	80,000 I.U.

Riboflavin (#54)	100,000 micrograms
Magnesium sulfate	15 grams

Thirty hens and four roosters were kept in a straw loft, open front, laying house. These birds were fed the basal ration supplemented with 300,000 I.U. of vitamin A per hundred pounds of feed for approximately three months previous to the beginning of this study. Fresh feed was prepared every 10 - 12 days. Crushed oyster shell and fresh water were available at all times.

This basal ration supplemented with vitamin A was considered adequate for good laying and for hatchability of fertile eggs, as judged by the reports of McClary et al. (33) and Bearse and Miller (34). A hatchability trial was conducted on eggs from this flock while they were being fed the experimental ration. It was found that 92 percent of the fertile eggs hatched, verifying the adequacy of the ration for hatchability.

Eggs were collected daily from February 19, 1949, to March 13, 1949, by trapnesting. Eggs were pedigreed by writing the hens' leg band numbers and the dates laid on the shells.

In order that information on the level of vitamin A in the eggs could be obtained, the first, fourth, sixth, eighth, etc., eggs of a laying cycle[1] were analyzed, whenever possible[2], as soon after laying as practical. The analyses were carried out,

[1]A laying cycle consists of the eggs laid on consecutive days by a particular hen; it is sometimes called a laying clutch.

[2]The number of eggs a hen laid in a cycle limited the extent to which the experimental design could be followed.

in all cases, within seventy-two hours after laying. In case the analysis could not be made immediately, the eggs were stored under refrigeration at 4° C. The second, third, fifth, seventh, etc., eggs of a laying cycle were incubated, whenever possible[2], for six, twelve, eighteen or twenty-one days. The analyses of incubated eggs were performed, in all cases, the same day they were removed from the incubator.

Fresh eggs, incubated infertile eggs, embryos (also the livers of the 18-and 21-day old embryos) and their residual yolk sacs were analyzed for total vitamin A content.

Upon completion of the foregoing phase of the study it was thought that additional valuable information could be obtained by repeating the work at both a lower and a higher level of vitamin A intake by the hen. Therefore, eight of the thirty hens used in the previous work were placed in laying batteries on April 7, 1949. Four of the hens received the basal ration without vitamin A supplementation until April 22, 1949. It was considered that these hens were sufficiently depleted at the latter date so that the vitamin A deposited in their eggs would come principally from the feed rather than from body stores. These hens were fed the basal ration supplemented with vitamin A at a level of 150,000 I.U. per hundred pounds of feed from April 22, 1949, until the end of the trial, June 4, 1949. The other four hens were maintained on the basal ration supplemented with 1,200,000 I.U. per hundred pounds of feed from April 7, 1949, until June 4, 1949.

The collection of eggs from the aforementioned eight hens began May 1, 1949, at which time it was thought that the eggs from the two groups of hens were fairly well stabilized at a lower and higher level, respectively, than were the eggs used in the first phase of this study. The fertility of the eggs from these hens was maintained by artificial insemination every third day.

The same methods of collection, pedigreeing, incubation, analysis, etc., were used with eggs from these two groups of hens as with those used in the first phase of the study.

Methods of analysis. A method of analysis for total vitamin A content of fresh egg yolk was developed by Neff (35) and was used without modification in this study. The procedure was as follows: The egg shell was cracked and as much of the albumen as possible drained. The yolk was dried by rolling on a towel in order to remove adherring egg white and transferred, without breaking the membrane, to a small funnel, supported in a beaker. The whole was weighed and, since the weight of the funnel and beaker had been previously determined, the yolk weight was determined by difference. The funnel containing the unbroken yolk was placed over a glass-stoppered, graduated mixing cylinder, which contained approximately 10 ml of 18 percent sodium chloride solution. The yolk membrane was broken and its contents allowed to drain into the cylinder. The presence of the salt solution in the cylinder aided in preventing the yolk from adherring to the bottom and walls of the container. The funnel and beaker contain-

ing the residue, essentially the yolk membrane, again was weighed and the sample weight obtained by difference. Sufficient 18 percent salt solution was added so that three ml of the final volume contained one gram of yolk.

The cylinder was stoppered and shaken until a homogenous emulsion was formed. A six-ml aliquot of this yolk emulsion was transferred by pipette to a 125 ml boiling flask and eight ml of alcoholic potassium hydroxide solution[3] was added. The mixture was refluxed for twenty minutes, after which it was transferred to a 250 ml separatory funnel. The transfer was made quantitatively by rinsing the flask with eight ml of 95 percent ethanol followed by a rinsing with eight ml of water. The rinsings were added to the separatory funnel.

The process used for extraction of the vitamin A was that of Boyer et al. (36) as modified by Neff (35). To the saponified sample in the separatory funnel was added 30 ml of cold diethyl ether and the funnel was shaken for two minutes. After separation into two phases, the lower one was drained into a second separatory funnel and re-extracted with 20 ml of ether by shaking for one minute. The extracts were washed three times successively with 60 ml of cold water, 30 ml of cold acidified alcoholic wash solution,[4] and 20 ml of cold acidified alcoholic wash solution.

[3]Prepared by dissolving 20 grams of potassium hydroxide in ten ml of water and adding 100 ml of 95 percent ethanol, Boyer et al. (36).

[4]Prepared by adding 100 ml of 95 percent ethanol to one ml of concentrated hydrochloric acid and diluting with water to one liter, Boyer et al. (36).

Each wash solution was discarded as it was drawn from the second separatory funnel. After the second washing with acidified alcoholic wash solution, the two extracts were combined in the first separatory funnel.

To the combined extracts, five ml of purified Skellysolve B were added and the extracts rewashed with 50 ml of cold water. The Skellysolve B served to decrease the solubility of the water in the ether, Boyer et al.(36). The extracts were allowed to stand for fifteen minutes in order to complete the separation of the two phases before the water was drained.

During these washing operations a tendency toward emulsification sometimes was evidenced. If an emulsion formed, usually it was broken by the addition of a few milliliters of ethanol and/or a little sodium chloride; however, brief centrifugation was necessary at times to effect a complete separation of the phases.

The ether extracts were evaporated in an all-glass assembly using reduced pressure and a hot (65-70°C) water bath. The cooled residue was dissolved in redistilled Skellysolve F and diluted to a volume convenient for the photometric determinations. A ten-ml aliquot was transferred to a photometric adsorption tube. The concentration of carotenoid pigments was determined with the Evelyn photometer using the 440 mμ filter.

The Skellysolve F solution was evaporated to dryness in the Evelyn tube using reduced pressure and a hot water bath. The residue was cooled and redissolved in one ml of chloroform. The

Carr-Price (22) antimony trichloride reagent was used for determination of vitamin A content. Nine milliliters of this reagent was added from the fast-flowing Parrish-Caldwell (37) pipette to the one ml of chloroform solution and the depth of the transient blue color determined on the Evelyn photometer using the 620 mμ filter. The galvanometer was read approximately five seconds after the addition of the Carr-Price reagent. Interference by carotenoids was corrected by using the factor 0.135, as determined by Neff (35).

Neff (35) has shown that vitamin A is not destroyed in this process since he was able to recover 101-109 percent of added vitamin A.

In the present investigation, determinations on duplicate samples indicated that good precision was attainable by this method. The average deviation from the average was 1.7 percent. The maximum deviation was 2.2 percent. Neff (35) obtained a precision of 1.4 percent average deviation from the average, with a maximum deviation of 2.5 percent.

A third extraction was performed on a number of the saponified samples in order to determine the completeness of the extraction of vitamin A. The third extraction recovered only 0.05 percent of the total vitamin A. It was considered, therefore, that the extraction was complete.

A blank analysis of the reagents indicated a galvanometer reading equivalent to 0.29 micrograms of vitamin A per two-gram sample. This artifact was subtracted from the vitamin A values

so that the data are indicative of true values within the limits of the method.

In the case of infertile eggs, analyses for total vitamin A were performed after incubation for 6, 12, 18, and 21 days. Since it was difficult to separate the yolk and white of incubated eggs, certain modifications of the procedure were necessary, as follows: The shell was broken and the contents emptied into a 250 ml boiling flask. No attempt was made to separate the white from the yolk due to the fragility of the yolk membrane after incubation. Eighty milliliters of alcoholic potassium hydroxide solution was added and the whole refluxed for thirty minutes.

Total digestion of the material usually was effected, especially if the flask was agitated as the saponification mixture slowly was added. Occasionally rubbery-like masses of coagulated protein were formed. Some of these masses were cut up, resaponified, and analyzed for vitamin A; however, no measurable amounts were found.

The saponified mixture was quantitatively transferred to a separatory funnel and extracted as previously discussed for fresh eggs, except that a further modification consisted of tripling the quantities of extractive and washing reagents.

The procedure was modified in the case of fertile eggs as follows: A hole was made in the shell at the air space and sufficient shell removed to allow for the withdrawal of the embryo. This and the succeeding operations were performed over a funnel

set in an appropriate boiling flask. A pair of forceps was introduced through the hole in the shell and the embryo removed. In the case of 6- and 12-day old embryos the extra-embryonic circulatory system tore loose and remained in the shell. It was found necessary to cut the residual yolk sacs from the 18-day embryos. The 21-day embryo had only a little solid matter left in the shell outside its body; no vitamin A was found to be present upon analysis of this material. The residual yolk sac of the 21-day old embryo was contained within its body and could be removed only by making a small incision at the point of fusion of the midgut. The yolk sac was removed by massaging the sides of the abdomen, Temperton et al.(33).

The embryo, after removal from the shell, was placed in sufficient 10 percent salt solution to cover it, which served to rinse off any adherring fluid. This solution was added to the material remaining in the shell after the removal of the embryo. This extra-embryonic material was transferred to a boiling flask to which was added 30, 60, 30 and 10 ml of alcoholic potassium hydroxide, respectively, for the saponification of 6-, 12-, 18-, and 21-day old extra-embryonic material. Care was taken in transferring this material since analyses indicated that the watery-like substance immediately surrounding the embryo was of a high vitamin A content. After refluxing this extra-embryonic material for thirty minutes, the extraction and washing procedure was carried out as stated above for fresh eggs, but modified by using the following quantities of extractive and washing reagents: The

first extraction of the residual material of 6, 12, 18 and 21 days of incubation was made, respectively, with 80, 60, 40 and 30 ml of cold diethyl ether. The second extraction of the residual material of 6, 12, 18 and 21 days of incubation was made, respectively, with 60, 40, 30 and 30 ml of cold ether. The first washing of the residual material of 6, 12, 18 and 21 days of incubation was made, respectively, with 80, 60, 40 and 30 ml of cold acidified alcoholic wash solution. The second washing of the residual material of 6, 12, 18 and 21 days of incubation was made, respectively, with 60, 40, 30 and 30 ml of cold acidified alcoholic wash solution. The extracts were combined and 120, 90, 60 and 60 ml of cold water and 20, 15, 10 and 8 ml, respectively, of purified Skellysolve B (to reduce the water content) were added to the extracts. The procedure from this point on was that described above for fresh eggs.

The vitamin A content of 6- and 12-day old embryos was determined by placing them in boiling flasks to which was added 10 and 30 ml, respectively, of alcoholic potassium hydroxide. After refluxing for thirty minutes the material was quantitatively transferred to a separatory funnel and extracted by the same method as stated above for fresh eggs. The quantities of extraction and wash reagents for the six-day old embryos were the same as those used for a two-gram sample of fresh egg yolk. Double quantities of extractive and wash reagents were used for the 12-day old embryos.

The livers of the 18- and 21-day embryos were removed by dissection and refluxed in boiling flasks for thirty minutes with 15 ml of alcoholic potassium hydroxide. The quantities of extractive and wash reagents used were the same as those for a two-gram sample of fresh egg yolk, as stated above.

The carcasses of the 18- and 21-day embryos, after removal of the livers, were cut into small pieces and saponified in boiling flasks with 70 and 90 ml, respectively, of alcoholic potassium hydroxide for thirty minutes. The extractive and wash reagents used were as follows: For the first ether extraction, 80 and 100 ml, respectively; for the second ether extraction, 50 and 70 ml, respectively; for the first water wash, 80 and 100 ml, respectively; for the first acidified alcoholic wash, 80 and 100 ml, respectively; for the second acidified alcoholic wash, 60 and 80 ml, respectively. The two extracts of each embryo were combined and 100 ml of water added. Fifteen milliliters of purified Skellysolve B were added to reduce the water content of the extracts. The procedure from this point on is that for fresh eggs.

The saponification of the carcasses did not dissolve the bones or feathers, however, it was believed that these contained no appreciable amounts of vitamin A.

The calculation of the amount of vitamin A in a sample was made by use of the following formula:

$$\text{Micrograms vitamin A per sample} = (L_{620} - 0.135 \times L_{440}) \times 13.1$$

where L designates the optical density of the solution for light of wave lengths 620 mμ and 440 mμ, respectively, and 0.135 is the correction factor for carotenoid interference. The L-value is obtained by looking up corresponding galvanometer readings in a table supplied with the photometer. If such a table is not available the L value may be obtained from the following formula:

$$L = 2 - \log G$$

where G equals the galvanometer reading.

If the final volume of the extract of the sample in Skellysolve F was 25 ml and a ten-ml aliquot of this was used in the analysis, obtaining the following galvanometer readings:

$$G_{440} = 91$$
$$G_{620} = 61$$

then

$$L_{440} = 0.0410$$
$$L_{620} = 0.2147$$

and the following calculations apply:

$$\text{Micrograms vitamin A} = (0.2147 - 0.135 \times 0.0410) \times 13.1 = 2.74.$$

Since a ten-ml aliquot of a 25-ml volume of the extract containing the sample was used, the value, 2.74 micrograms, was corrected for the total sample by multiplying by 25/10, thus, the final value was 6.86 micrograms per sample.

Experimental Results

The variations in the vitamin A content of chick embryos from eggs of hens receiving the ration supplemented with vitamin A at low, medium and high levels are presented in Tables 1, 2 and 3.

These data show that there is a marked increase in vitamin A content with increase of embryonic age. Embryos from eggs of hens receiving the diet supplement with vitamin A at a low level (150,000 I.U. per hundred pounds of feed) contained 0.186, 1.20, 2.34 and 3.10 micrograms of vitamin A, respectively, at 6, 12, 18 and 21 days of age. Embryos from eggs of hens receiving the diet supplemented with vitamin A at a medium level (300,000 I.U. per hundred pounds of feed) contained 0.245, 1.96, 13.6 and 18.4 micrograms of vitamin A, respectively, at 6, 12, 18 and 21 days of age. Embryos from eggs of hens receiving the diet supplemented with vitamin A at a high level (1,200,000 I.U. per hundred pounds of feed) contained 0.379, 2.42, 15.0 and 30.6 micrograms of vitamin A, respectively, at 6, 12, 18 and 21 days of age.

These data are graphically interpreted in Figs. 1 and 2. Fig. 1 is the graph of the embryonic vitamin A content, in micrograms, plotted against the days of incubation, i.e., embryonic age. This graph indicates that an increase in the vitamin A content of the feed a hen receives caused an increase in the vitamin A content of the embryos in that hen's eggs. The increase in the embryonic vitamin A content is not proportional to the intake of vitamin A by the hen, since doubling the vitamin A

Table 1. The vitamin A content of chick embryos of various ages in eggs from hens on a vitamin A supplemented diet*.

Hen number from which eggs were obtained	Embryonic age in days			
	6 days	12 days	18 days	21 days
	micrograms per embryo			
3978**	--	--	--	--
3991	0.279	1.80	3.66***	2.76***
9410	0.096	0.62	1.29	3.40
	0.0****	--	--	3.60
	--	--	--	2.22
9430	0.263	1.19	2.06	3.50
	0.291	--	--	--
Mean	0.186	1.20	2.34	3.10
Standard Deviation	± 0.13	± 0.59	± 1.2	± 0.53

*Ration supplemented with vitamin A at a level of 150,000 I.U. per hundred pounds of feed.

**Hen number 3978 laid irregularly during this study and the data were not collected.

***Liver and carcasses for the 18 and 21 day embryos were determined separately. The values shown are totals obtained by addition.

****The artifact cancelled the vitamin A value on this determination.

Table 2. The vitamin A content of chick embryos of various ages in eggs from hens on a vitamin A supplemented diet.*

Hen number from which eggs were obtained	Embryonic age in days			
	6 days	12 days	18 days	21 days
	micrograms per embryo			
9424	0.206	1.92	13.2**	----**
3986	0.239	1.96	----	----
3989	0.299	1.89	14.3	18.5
	----	1.88	----	----
3991	0.280	----	12.9	16.0
	----	2.02	12.5	----
	----	1.73	----	----
3976	0.333	1.46	----	----
9410	0.175	1.80	15.9	16.4
	----	----	15.0	----
9420	0.259	2.13	13.6	19.7
9422	0.069	----	----	----
3974	0.309	2.59	11.9	20.8
	----	----	15.0	20.7
9404	0.253	2.00	----	----
9428	0.336	2.04	11.9	19.9
	----	----	13.8	----
9429	0.095	----	----	----
9419	----	----	16.5	----
	----	----	15.4	----
3978	0.261	1.92	13.4	15.1
9430	0.311	2.15	9.1	----
Mean	0.245	1.96	13.6	18.4
Standard deviation	± 0.083	±0.25	± 1.9	± 2.25

*Ration supplemented with vitamin A at a level of 300,000 I.U. per hundred pounds of feed.

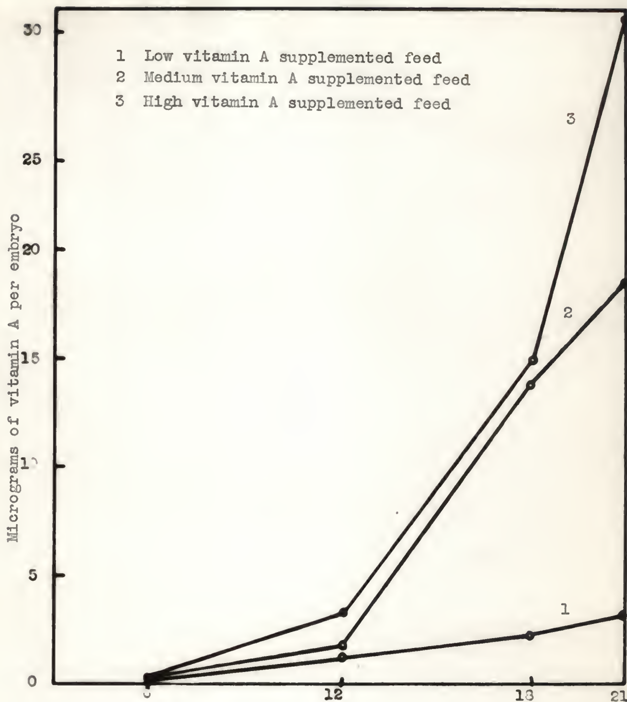
**Liver and carcasses for the 18 and 21-day embryos were determined separately. The values shown are totals obtained by addition.

Table 3. The vitamin A content of chick embryos of various ages in eggs from hens on a vitamin A supplemented diet.*

Hen number from which eggs were obtained	Embryonic age in days			
	6 days	12 days	18 days	21 days
	micrograms per embryo			
9420	0.616	1.81	17.3**	29.0**
	----	2.91	----	----
9428	0.271	1.89	18.8	24.9
3974	0.492	3.20	11.2	37.1
	----	1.94	----	----
3989	0.136	2.94	12.6	31.4
	----	2.23	----	----
Mean	0.379	2.42	15.0	30.6
Standard Deviation	±0.22	±0.58	±3.66	±5.0

*Ration supplemented with vitamin A at a level of 1,200,000 I.U. per hundred pounds of feed.

**Liver and carcasses for the 18- and 21-day embryos were determined separately. The values shown are totals obtained by addition.



Days of incubation, i.e., embryonic age

Fig. 1. Graphical representation of data from Tables 1,2, and 3. Embryonic age plotted against vitamin A content of embryos from eggs of hens on feed supplemented with vitamin A at three levels.

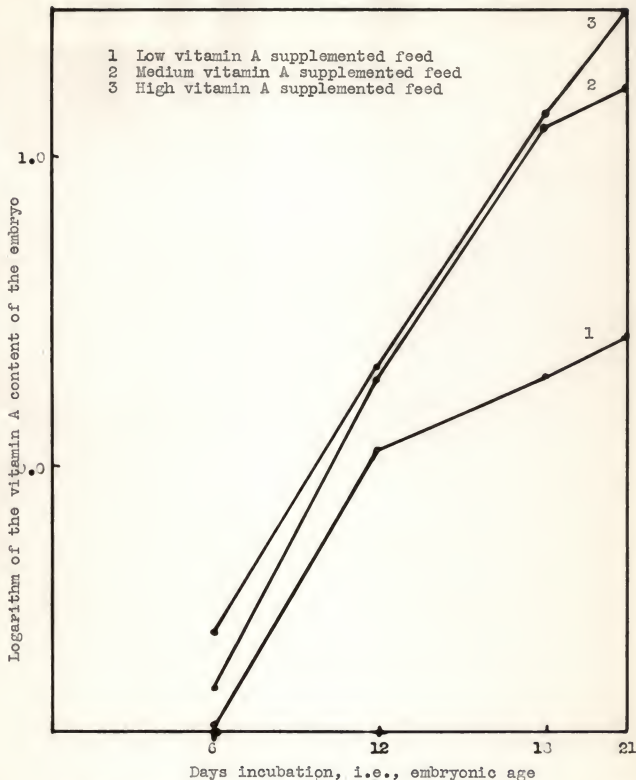


Fig. 2. Graphical representation of data from Tables 1,2, and 3. Embryonic age plotted against the logarithm of vitamin A content of embryos from eggs of hens on feed supplemented with vitamin A at three levels.

level of the feed caused a sixfold increase of vitamin A in the 21-day old embryo as compared to that of the embryo from the egg of the hen on the lower level; whereas, an eightfold increase in the vitamin A content of the feed caused only about ten times as much vitamin A to appear in the embryo.

Fig. 2 is a second graphical representation of the data contained in Tables 1, 2 and 3. The logarithm of the vitamin A content of the chick embryo, is graphed versus the embryonic age. From this graph it appears that the vitamin A content of the chick embryo approaches a straight line relationship when the maternal hen received vitamin supplementation at the highest level. At the low level of vitamin intake by the hen, sufficient quantities of this vitamin were deposited in the egg so that a straight line relationship was found through only the twelfth day of embryonic age. Supplementation at the medium level supplied sufficient vitamin A so that this relationship continued until the eighteenth day of embryonic age.

Data were obtained on the vitamin A content of eggs, the loss of vitamin A during incubation (both fertile and infertile eggs), relation of the embryonic vitamin A content to the total vitamin A content, the relation of the residual yolk sac vitamin A to the total vitamin A present, and the relationships between the vitamin A content of the embryo, its liver and residual yolk sac. These data were obtained only on eggs from hens receiving the ration supplemented with vitamin A at a medium level (300,000

I.U. per hundred pounds of feed).

Table 4. contains the data obtained from the analysis of unincubated eggs, together with the graphical estimation of the amount of vitamin A in the incubated eggs that might have been expected in the fresh eggs had they not been incubated. It was necessary to arrive at an estimation of the vitamin A content of these eggs in order that the loss of vitamin A during incubation could be determined. The method of arriving at these estimations is illustrated in Fig. 3. The vitamin A content (obtained by analysis) of certain of the eggs of a laying cycle were plotted against the number of the egg in the cycle, Neff (35). The probable vitamin A content of the eggs which were not analyzed were obtained by interpolation. This method does not lead to extremely accurate estimates, but gives an indication of the general level of vitamin A in the egg before incubation.

Table 5. presents the data on the amount and percent of vitamin A lost from the fertile egg during incubation. These data are based on the probable amount of vitamin A present in the egg at the beginning of incubation, as shown in Table 4, and the sum of the vitamin A content of the embryo and its residual yolk sac. The data indicate that during incubation the fertile egg lost 6.6 micrograms (9.3 percent), 7.8 micrograms (11.7 percent), 15.9 micrograms (22.6 percent), and 18.9 micrograms (27.8 percent), respectively, when incubated for 6, 12, 18 and 21 days.

Table 4. The vitamin A content of eggs from hens on the ration supplemented with vitamin A at a medium level (300,000 I.U. per hundred pounds of feed).

Hen no.	Egg in laying cycle	Vit. A per egg	Vit. A per egg graphical interpol'n##
		micrograms	
9422	1	63.4	----
	2*	----	60#
3986	1	93.2	----
	2*	----	90#
	3*	----	85#
3991	1	59.3	----
	2*	----	56#
	3*	----	52#
3976	1	72.6	----
	2*	----	69#
	3*	----	65#
	1**	78.8	----
	2*	----	75#
	3*	----	70#
	1**	73.4	----
9410	1	77.5	----
	2*	----	70#
	3*	----	65#
	1**	53.6	----
9420	1	71.8	----
	2*	----	68#
	3*	----	63#
	1**	62.1	----
9419	1	62.5	----
	2*	----	58#
	3*	----	55#
	1**	59.0	----
3978	1***	----	68#
	2*	----	63#
	3	60.3	----
3989	1	68.4	----
	2*	----	67
	3*	----	67
	4	65.9	----
3976	1	88.5	----
	2*	----	83
	3*	----	79
	4	74.8	----
	1**	72.6	----
9429	1	80.1	----
	2*	----	75
	3***	----	71
	4	66.1	----
	1**	70.9	----
	2*	----	71
	3*	----	71
	4	72.0	----
9430	1	53.8	----
	2*	----	52
	3*	----	50
	4	49.0	----
	5*	----	51
	1**	54.0	----
9424	1	73.3	----
	2*	----	71
	3*	----	69
	4	67.2	----
	5*	----	64
	1**	62.0	----
9410	1	53.6	----
	2*	----	52
	3*	----	50
	4	48.5	----
	5*	----	52
	1**	54.0	----

Table 4. (con'td)

Hen no.	Egg in laying cycle	Vit. A per egg	Vit. A per egg graphical interpol'n##
9420	1	62.1	----
	2*	----	64
	3*	----	66
	4	68.2	----
	5*	----	70
3974	1	93.4	----
	2*	----	89
	3*	----	85
	4	81.6	----
	5*	----	77
9419	1	56.8	----
	2*	----	59
	3*	----	62
	4	64.3	----
	5*	----	63
	1**	62.5	----
3978	1	79.2	----
	2*	----	75
	3*	----	71
	4	66.7	----
	5*	----	63
3989	1	83.5	----
	2*	----	82
	3*	----	80
	4	78.5	----
	5*	----	73
	6	68.9	----
	1**	68.4	----
3974	1	86.8	----
	2*	----	88
	3*	----	89
	4***	----	90
	5*	----	91
	6*	----	93
	1**	93.4	----
9428	1	70.3	----
	2*	----	68
	3*	----	66
	4	64.6	----
	5*	----	64
	6	63.9	----
	1**	60.0	----
9404	1	55.4	----
	2*	----	55
	3*	----	55
	4***	----	55
	5	55.0	----
	6*	----	52
	7	49.6	----
	8****	----	45
	1**	45.1	----
3991	1	61.9	----
	2*	----	62
	3	----	62
	4	62.4	----
	5*	----	61
	6	59.9	----
	7*	----	55
	8	50.6	----
	9*	----	55
	1**	59.3	----
3972	1	60.4	----
	2*	----	60
	3*	----	60
	4	59.6	----
	5*	----	58
	6	56.9	----
	7*	----	57
	8	57.1	----
	9*	----	57
	10	57.9	----
	11*	----	59

Table 4. (concl.)

Hen no.	Egg in laying cycle	Vit. A per egg	Vit. A per egg graphical interpol'n ##
3972	12	59.8	----
	13*	----	58
	14	55.8	----
	15*	----	57
	16	58.6	----
	17*	----	60

*Egg incubated.
**First egg of next laying cycle.
***Egg lost during analysis.
****Dead embryo of indeterminate age.
#Value estimated, due to lack of data.
##Values were arrived at by graphical interpolation method, see text.

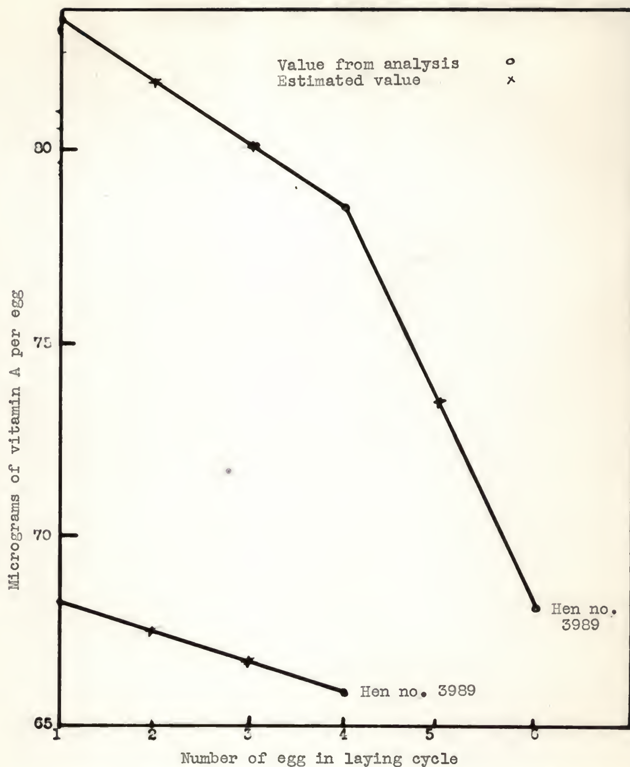


Fig. 3. Method of arriving at an estimate of the vitamin A content of an incubated egg that might have been had the egg not been incubated.

Table 5. The amount and percent of vitamin A lost from the fertile egg during incubation, data from eggs of hens on the diet supplemented with vitamin A at a medium level.*

Hen no.	Probable am't of vit. A in the egg***	Am't of vit A in embryo***	Am't of vit. A in yolk sac	Am't of vit. A lost from egg	Vit. A lost from egg
	micrograms				Percent
6-day old embryos					
9424	71	0.206	53.2	17.6	24.8
3986	90	0.239	81.0	8.8	9.5
3989	82	0.299	71.6	10.1	12.5
3991	60	0.280	53.4	6.3	10.5
3976	83	0.333	77.0	5.7	6.9
9410	70	0.175	51.4	18.4	26.3
9420	68	0.259	60.2	7.5	11.0
9422	60	0.069	49.3	10.6	17.5
3974	88	0.309	86.2	1.5	1.8
9404	55	0.253	54.3	0.4	0.9
9428	68	0.336	70.6	-2.9	-4.1
9429	75	0.095	71.0	3.9	5.2
3978	75	0.261	71.0	3.7	4.9
9430	52	0.311	50.7	1.0	2.0
MEAN		0.245		6.6	9.3
12-day old embryos					
9424	--	----	----	----	----**
3986	85	1.96	91.6	-8.6	-10.0
3989	80	1.89	53.8	24.3	30.4
3989	67	1.88	52.0	13.1	19.6
3991	56	2.02	41.3	12.7	22.8
3991	52	1.73	47.6	2.7	5.2
3976	79	1.46	69.2	8.2	10.5
9410	65	1.80	43.9	19.3	29.5
9420	63	2.13	55.5	5.4	8.6
3974	85	2.59	73.6	8.8	10.3
9404	55	2.00	46.8	6.2	11.2
9428	66	2.04	60.1	3.9	5.7
3978	71	1.92	64.0	5.1	7.2
9430	50	1.96	47.6	0.4	0.9
MEAN		1.96		7.8	11.7
18-day old embryos					
9424	64	13.2	34.5	17.3	27.1

Table 5. (concl.)

Hen no.	Probable am't of vit. A in the egg***	Am't of vit. A in embryo****	Am't of vit A in yolk sac	Am't of vit. A lost from egg	Vit. A lost from egg
3989	73	14.3	14.3	17.4	23.2
3991	61	12.9	32.7	15.4	25.3
	55	12.5	27.8	14.7	26.8
9410	52	15.9	31.1	5.0	9.5
	52	15.0	23.0	14.0	27.0
9420	70	13.6	29.0	37.4	39.1
3974	91	11.9	58.0	21.1	23.2
	89	15.0	68.2	5.8	6.5
9428	64	11.9	36.1	16.0	25.0
	62	13.8	34.1	14.1	22.9
9419	63	16.5	39.2	7.3	11.8
	55	15.4	35.6	4.0	7.3
3978	63	13.4	24.5	25.1	39.8
9430	51	9.1	18.3	23.6	46.3
MEAN		13.6		15.9	22.6
21-day old embryos					
3989	67	18.5	37.3	11.2	16.7
3991	55	16.0	18.8	20.2	36.7
9410	50	16.4	23.4	10.2	20.4
9420	66	19.7	29.1	17.2	26.1
3974	93	20.8	37.8	34.4	37.0
	77	20.7	35.0	21.3	27.8
9428	62	19.9	37.0	5.1	8.3
3978	63	15.1	16.8	31.1	49.3
MEAN		18.4		18.9	27.8

*Ration supplemented with vitamin A at a level of 300,000 I.U. per hundred pounds of feed

**The yolk sac was lost during analysis therefore no data was available.

***Data has already been presented in Table 4.

****Data has already been presented in Table 2.

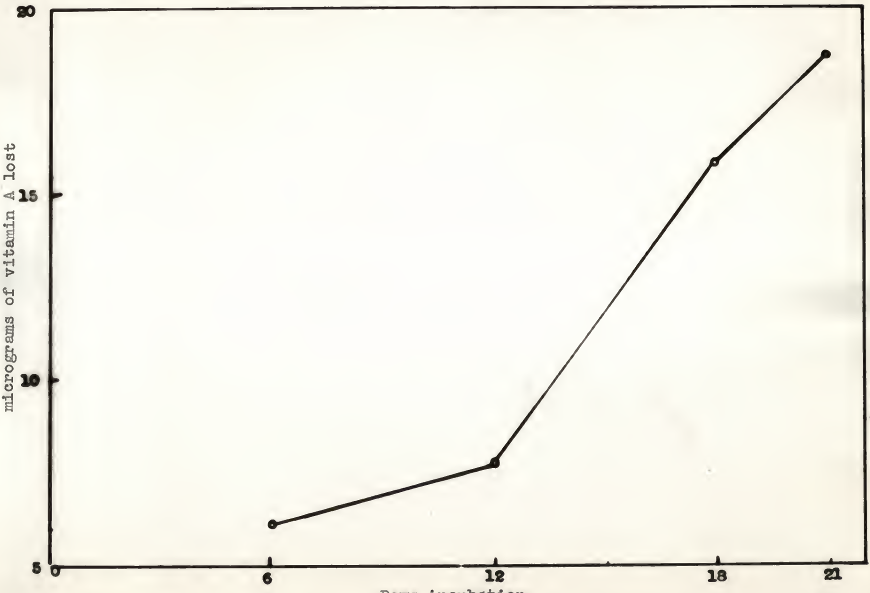


Fig. 4. Data from Table 5, graphically represented by plotting the vitamin A lost from the fertile egg during incubation against the days of incubation.

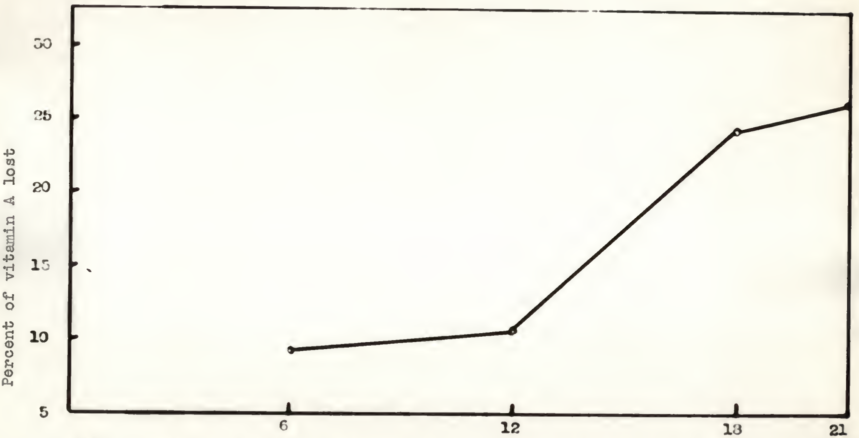


Fig. 5. Data from Table 5, graphically represented by plotting the percent vitamin A lost during incubation from the fertile egg versus days of incubation.

Losses of vitamin A from the egg during incubation are represented graphically in Fig. 4 and 5. Fig. 4 is the graph of the amount of vitamin A lost during incubation plotted against the days of incubation. Fig. 5 is the graph of the percent of vitamin A lost during incubation plotted against the days of incubation.

From these data and their graphical representations it is evident that the amount of vitamin A lost from the fertile egg during incubation increases as the length of the period of incubation increases.

Table 6 presents the data on the percent of vitamin A in the egg at the beginning of incubation which was present in the embryo after 6, 12, 18 or 21 days, and of the percent of vitamin A in the egg at the beginning of incubation which is present in the residual yolk after 6, 12, 18 and 21 days of incubation. The vitamin A values at the beginning of incubation were the estimations from Table 4.

There was present in the embryo 0.351, 2.98, 21.7 and 28.1 percent of the vitamin A probably present at the beginning of incubation, after 6, 12, 18 and 21 days of incubation, respectively. The residual yolk sac of the 6, 12, 18 and 21-day old embryos contained, respectively, 90.4, 86.3, 54.3 and 45.9 percent of the probable amount of vitamin A present in the egg at the time of incubation.

Table 7 contains the data on the relationship between the vitamin A content of the embryo and its liver, and between the liver and residual yolk sac.

Table 6. Some relationships between the probable vitamin A content of the egg before incubation and the vitamin A content of the embryo and residual yolk sac, data from eggs of hens on the diet supplemented with vitamin A at a medium level.*

Hen no.	Am't of vit. A in embryo***	Am't of vit. A in yolk sac****	Probable am't of vit. A in the egg *****	Probable vit. A of egg, present in embryo	Probable vit. A of egg, present in yolk sac.
	micrograms			percent	
6-day old embryos					
9424	0.206	53.2	71	0.291	75.1
3986	0.239	81.0	90	0.265	89.9
3989	0.299	71.6	82	0.364	87.1
3991	0.280	53.4	60	0.466	89.0
3976	0.333	77.0	83	0.402	92.7
9410	0.175	51.4	70	0.250	73.5
9420	0.259	60.2	68	0.380	88.8
9422	0.669	49.3	60	0.115	82.1
3974	0.309	86.2	88	0.350	98.2
9404	0.253	54.3	55	0.460	98.6
9428	0.336	70.6	68	0.495	103.8
9429	0.095	71.0	75	0.127	94.6
3978	0.261	71.0	75	0.349	94.6
9430	0.311	50.7	52	0.599	97.3
MEAN				0.351	90.4
12-day old embryos					
9424	----	----	--	-----	----**
3986	1.96	91.6	85	2.30	108.0
3989	1.89	53.8	80	2.36	67.1
	1.88	52.0	67	2.80	77.5
3991	2.02	41.3	56	3.60	73.9
	1.73	47.6	52	3.33	91.5
3976	1.46	69.2	79	1.85	87.6
9410	1.80	43.9	65	2.76	67.5
9420	2.13	55.5	63	3.38	88.0
3974	2.59	73.6	85	3.04	86.5
9404	2.00	46.8	55	3.64	84.9
9428	2.04	60.1	66	3.09	90.9
3978	1.92	64.0	71	2.70	90.0
9430	1.96	47.6	50	3.92	95.1
MEAN				2.98	86.3

Table 6. (concl).

Hen no.	Am't of vit. A in embryo	Am't of vit. A in yolk sac	Probable am't of vit. A in the egg	Probable vit. A of egg, present in embryo	Probable vit. A of egg, present in yolk sac
18-day old embryos					
9424	13.2	34.5	64	20.6	53.9
3989	14.3	41.3	73	19.6	56.6
3991	12.9	32.7	61	21.2	53.8
	12.5	27.8	55	22.7	50.2
9410	15.9	31.1	52	30.6	59.9
	15.0	23.0	52	28.9	44.3
9420	13.6	29.0	70	19.4	41.4
3974	11.9	58.0	91	13.1	63.8
	15.0	68.2	89	16.8	77.0
9428	11.9	36.1	64	18.6	56.6
	13.8	34.1	62	21.2	55.0
9419	16.5	39.2	63	26.1	62.2
	15.4	35.6	55	28.0	64.9
3978	13.4	24.5	63	21.3	38.8
9430	9.1	18.3	51	17.9	35.8
MEAN				21.7	54.3
21-day old embryos					
3989	18.5	37.3	67	27.6	55.6
3991	16.0	18.8	55	29.1	34.1
9410	16.4	23.4	50	32.8	46.7
9420	19.7	29.1	66	29.9	44.1
3974	20.8	37.8	93	22.4	40.6
	20.7	35.0	77	26.9	49.9
9428	19.9	37.0	62	32.2	59.8
3978	15.1	16.8	63	24.0	26.3
MEAN				28.1	45.9

*Ration supplemented with vitamin A at a level of 300,000 I.U. per hundred pounds of feed.

**The yolk sac was lost during analysis therefore no data was available.

***Data previously presented in Table 2.

****Data previously presented in Table 5.

*****Data previously presented in Table 4.

Table 7. Some relationships between the vitamin A content of the embryo and its liver, and between the liver and residual yolk sac, data from eggs of hens receiving the ration supplemented with vitamin A at the level of 300,000 I.U. per 100 pounds of feed.

No. of hen from which eggs were obtained	Vit. A per embryo*	Vit. A per liver	Vit. A per yolk sac**	Vit. A of embryo in liver	Vit. A of yolk sac in embryo
	micrograms			percent	
18-day old embryos					
9424	13.2	5.7	34.5	43.2	16.5
3989	14.3	5.4	41.3	37.7	13.1
3991	12.9	4.7	32.7	36.4	14.3
	12.5	5.2	27.8	41.6	18.7
9410	15.9	6.9	31.1	43.4	18.9
	15.0	6.5	23.0	43.3	28.3
9420	13.6	6.6	29.0	48.4	22.8
3974	11.9	6.3	58.0	52.9	10.8
	15.0	9.8	68.2	65.2	14.4
9428	11.9	4.8	36.1	40.3	13.2
9419	16.5	6.1	39.2	37.0	15.6
9428	13.8	5.5	24.1	39.7	16.1
9419	15.4	7.5	35.6	48.6	21.1
3978	13.4	6.2	24.5	46.2	25.3
9430	9.1	6.2	18.3	68.1	33.9
MEAN				26.3	19.2
3989	18.5	9.2	37.3	49.7	24.7
3991	16.0	5.5	18.8	34.4	29.3
9410	16.4	6.1	23.4	37.2	26.2
9420	19.7	9.4	29.1	47.6	32.2
3974	20.8	13.3	37.8	63.8	35.2
	20.7	14.9	35.0	71.9	42.5
9428	19.9	8.1	37.0	40.7	21.9
3978	15.1	5.6	16.8	37.2	33.3
MEAN				48.1	30.7

*Data previously presented in Table 2.
**Data previously presented in Table 5.

These data show that the percentage of vitamin A in the embryonic liver increases from 26.3 to 48.1 during the last three days of incubation, the change, expressed as percent, was from 19.2 to 30.7.

Table 8 presents the data on the loss of vitamin A by the infertile eggs during incubation. They lost 0.35 micrograms (1.7 percent), 11.6 micrograms (19.1 percent), 12.5 micrograms (19.6 percent), and 17.2 micrograms (27.6 percent) of vitamin A after 6, 12, 18 and 21 days of incubation.

Figs. 6 and 7 graphically represent data from Table 8. Fig. 6 is the graph of the micrograms of vitamin A lost plotted against the days of incubation.

Discussion

Upon examination of the data and their graphical representation it is evident that the vitamin A content of chick embryos increases with embryonic age. Furthermore, the vitamin A content of chick embryos in the later stages of development is dependent upon the initial vitamin A content of the egg. It appears from these data that if sufficient vitamin A is present initially in the egg, the transfer curve of this vitamin obtained when the logarithm of the vitamin A content of the embryo is plotted against the embryonic age, is a straight line. If lesser quantities of vitamin A are present initially in the egg, the straight line relationship falls off in the latter stages of development as the rate of transfer becomes smaller. Under the conditions of this study the upper limit was closely approached when the

Table 8. The amount and percent of vitamin A lost from the infertile egg during incubation, data from eggs of hens on the medium level vitamin A supplemented feed.*

Hen no. from which eggs were obtained	Probable am't of vit. A in the egg**	Am't in egg after incubation	Am't of vit. A lost from egg	Vit. A lost from egg
	micrograms			percent
6-day old embryos				
9419	59	64.7	-5.7	-9.1
3972	60	52.6	6.4	12.5
MEAN			0.35	1.7
12-day old embryos				
3991	62	54.8	7.2	11.9
9419	62	53.7	8.3	13.6
3972	60	41.6	18.4	30.6
	59	49.5	9.5	16.1
	57	42.0	15.0	26.3
3976	70	59.0	11.0	15.8
MEAN			11.6	19.1
18-day old embryos				
3976	69	56.8	12.2	17.9
	75	54.5	20.5	27.4
9420	64	54.6	9.4	14.9
9404	52	43.1	8.9	17.2
9429	71	61.1	9.9	14.0
3972	58	40.7	17.3	30.0
	57	48.0	9.0	16.0
MEAN			12.5	19.6
21-day old embryos				
3976	65	51.9	13.1	20.2
9428	71	57.0	14.0	20.0
3972	57	32.6	24.4	42.7
MEAN			17.2	27.6

*Feed supplemented at a level of 300,000 I.U. of vitamin A per hundred pounds of feed.
**Data previously presented in Table 4.

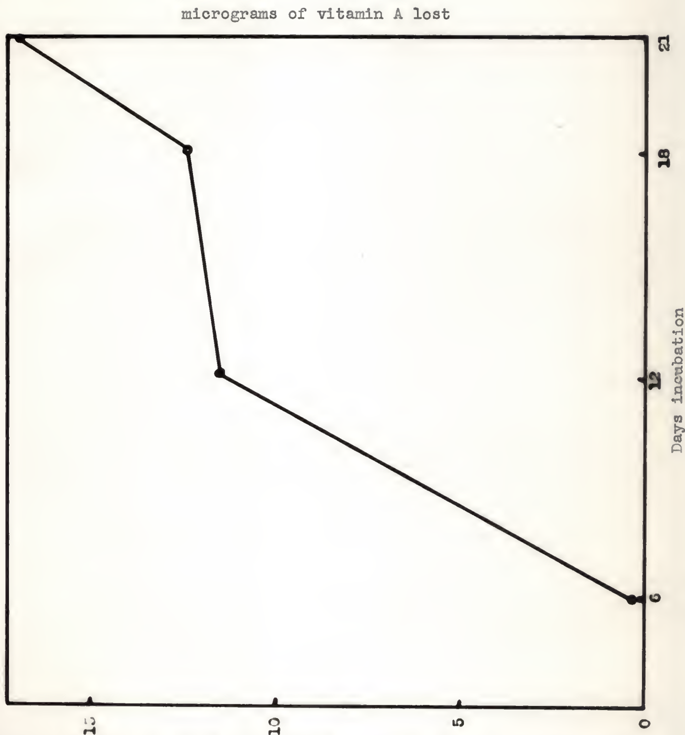


Fig. 6. Data from Table 8, graphically presented by plotting vitamin A lost from infertile egg against days of incubation.

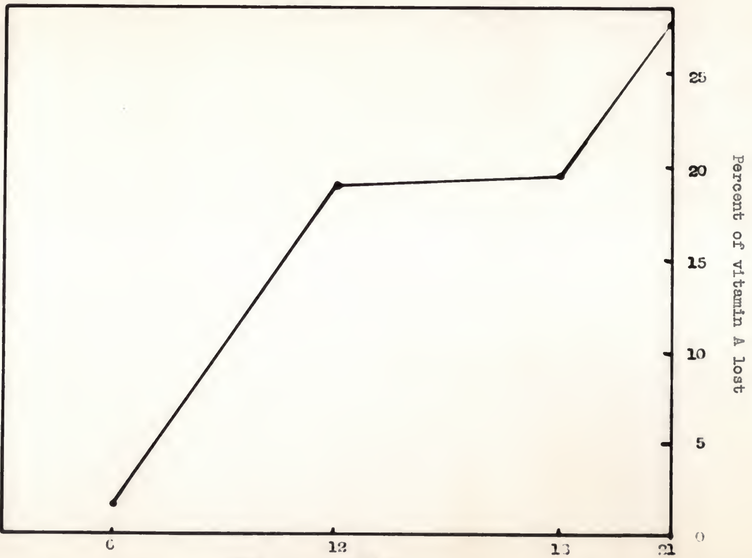


Fig. 7. Data from Table 8, graphically represented by plotting the percent of vitamin A lost from the infertile egg during incubation against days of incubation.

eggs were obtained from hens on a ration supplemented with vitamin A at a level of 1,200,000 I.U. per hundred pounds of feed. Further investigation along these lines is desirable to determine if this straight line relationship can be modified by massive concentrations of vitamin A in the egg.

Penquite (4) has indicated this same straight line relationship when the logarithm of wet weight, dry weight, or total nitrogen content of the embryo is graphed versus the embryonic age.

The exponential increase in the concentration of a particular nutrient in the chick embryo during its development appears to be a somewhat general rule of nature. The embryonic concentrations of total carbohydrate (5), certain of the amino acids (6), non-protein nitrogen (7), and fatty acids (8) seem to follow this exponential increase with embryonic age.

Scrimshaw et al. (11) has found a similar relationship in thiamine content of the chick embryo during development.

A decrease in the total vitamin A content of the egg, fertile and infertile, during incubation is indicated by the data. The percentage loss of vitamin A from infertile eggs after 21 days of incubation appears to be of the same order of magnitude as the loss of vitamin A from fertile eggs incubated for the same number of days. The present data indicate that after 6 and 18 days of incubation considerably more vitamin A appears to have been lost from the fertile egg than the infertile. It is questionable whether these are true differences; it is possible that they result from the paucity of data on the incubated infertile eggs.

If, however, the data on the absolute amount of vitamin A lost by the fertile and infertile eggs during incubation are compared, it is seen that there exists no marked differences in the losses. It would seem, therefore, that no vitamin A is destroyed or metabolized by the embryo during its development. However, a further and more extensive study of this particular problem should be made.

The data indicate that the liver is the principal storage organ for vitamin A in 18- and 21-day embryos.

An incidental finding of this study was the ability of chicks to hatch from eggs that were of lower vitamin A content than that reported in the literature. Lissot and Caridroit (16) have reported the chick embryo to require 350 I.U. of vitamin A for complete development and hatching, which is approximately 2-3 times higher than found in the present study. A re-investigation of the vitamin A requirements necessary for the development and hatching of the chick would seem to be indicated.

STATE OF VITAMIN A IN INFERTILE EGGS, IN EMBRYONIC CHICK LIVERS, AND IN RESIDUAL YOLK SAC

Procedure

Management of the experimental fowls. The eggs that were used for determination of the state of vitamin A were collected from four White Leghorn hens. The hens were maintained on the same pigment-low ration as the hens in the previous phases of this study.

These hens were housed in laying batteries. The basal ration

was supplemented with 1,200,000 I.U. of vitamin A per hundred pounds of feed. Fresh water and crushed oyster shell were available to the hens at all times. Fertility of the eggs was effected by artificial insemination.

Eggs were collected daily from May 1, 1949, to June 4, 1949. Most of these eggs were used to study variations in the vitamin A content of the chick embryo, as previously presented. The eggs from the last five days of the collection period were used for this phase of the study. The eggs were pedigreed by writing the hen's legband number and date laid on the shell. These eggs were placed in the incubator as soon after laying as possible. The eggs were removed from the incubator after either 18 or 21 days.

The liver of the embryo, the residual yolk sac, and the infertile eggs were analyzed for content of vitamin A alcohol and of vitamin A ester.

Methods of analysis. A method of analysis for the state of vitamin A in eggs was developed by Neff (39). A modification of this method was used for the determination of the state of vitamin A in incubated, infertile eggs, since the yolk membrane after incubation was very fragile and it was difficult to separate the white from the yolk.

An emulsion of the whole egg was prepared by emptying the egg into a 100 ml, glass-stoppered, mixing cylinder. Eighteen percent salt solution was added to the mark and the cylinder shaken to form an emulsion. A ten-ml aliquot of this emulsion was analyzed for total vitamin A content by the same method used for the determination of total vitamin A in fresh eggs, (page 8).

A second ten-ml aliquot of this emulsion was transferred to a glass-stoppered centrifuge tube.

The method from this point on is that of Neff (39). Eight milliliters of 95 percent ethanol was added and the tube shaken to mix thoroughly. Thirty milliliters of purified Skellysolve B was added and the tube shaken for three minutes to extract the fat-soluble material. Two additional extractions were made using 15 ml of purified Skellysolve B each time and shaking for two minutes. After each extraction the tube was centrifuged and the supernatant liquid transferred to a separatory funnel. The combined extracts were washed three times with cold water, using 70-, 50- and 50-ml quantities, respectively. Each wash solution was re-extracted in a second separatory funnel using 20 ml of purified Skellysolve B. The extracts were combined in the first separatory funnel. After complete separation of the two phases the water was drained and the extracts were placed in an all-glass assembly and evaporated to a volume of 10-15 ml using reduced pressure and a hot water bath (65-70°C.).

The method of preparation of the adsorption column was due to Parrish et al. (40). The column was prepared in a 200 by 12 mm glass tube, constricted at the lower end and joined to a short piece of six mm tubing. A 5-7 cm chromatographic column was formed by shaking the adsorbant, activated alumina, into the tube while a gentle vacuum was maintained. The column was supported by a small plug of cotton in the constricted end of the tube. The adsorbant was washed by drawing about 20 ml of purified

Skellysolve B through it under gentle vacuum. Just before the level of the Skellysolve B reached the top of the column the Skellysolve B solution of the lipids was added. The vitamin A esters were eluted from the column by use of about 90 ml of four percent acetone in Skellysolve B. The eluate was evaporated to 1-2 ml in a small boiling flask placed on a steam cone. Ten milliliters of alcoholic potassium hydroxide solution was added and the mixture refluxed for twenty minutes.

The extraction and photometric estimation of the vitamin A esters was that stated for total vitamin A of fresh eggs (page 9).

The livers and residual yolk sacs of the 18- and 21-day old embryos were analyzed by the method for livers of Parrish et al. (40, 41). The whole fresh liver or yolk sac, immediately after removal from the embryo, was ground with anhydrous sodium sulphate until a homogenous, mealy product was obtained. The tenderness of the embryonic liver made homogenization in the Waring Blendor unnecessary.

The lipids were extracted from the dried material by agitation in the Waring Blendor micro cup for four minutes with 20 ml of ethanol and 50 ml of chilled diethyl ether. This treatment was followed by three two-minute extrsctions with 30 ml of ether each time. The final extraction was made with 15 ml of Skellysolve B and 15 ml of ether. After each extraction the solids were separated from the extraction solvents by filtering through a fritted glass Buchner funnel under reduced pressure.

The extracts were combined in a separatory funnel and washed twice with cold water in an amount equivalent to that of the

extract. Care was taken in washing the extract since shaking often produced an emulsion. The addition of a little sodium chloride aided in breaking these emulsions; however, centrifugation was often necessary. The wash water was re-extracted using 30 ml of ether. The extracts were combined in the first separatory funnel and the water drained as completely as possible.

The combined extract was transferred to an all-glass evaporation assembly and the solvent evaporated by the use of reduced pressure and a hot water bath. The cooled residue was taken up in Skellysolve B and made to a convenient volume.

An aliquot of this solution was analyzed for total vitamin A by saponifying with ten ml of alcoholic potassium hydroxide solution for twenty minutes. The extraction and photometric procedure was that used for the total vitamin A content of fresh eggs (page 8).

A second aliquot of this solution was chromatographed and the vitamin A esters eluted from the column by the same method used for the separation of vitamin A alcohol and its esters of the incubated, infertile eggs. The eluate was evaporated to 1-2 ml in a small boiling flask heated on a steam cone. Ten milliliters of alcoholic potassium hydroxide was added and the mixture refluxed for twenty minutes. The extraction and photometric estimation of the vitamin A esters was that stated for total vitamin A of fresh eggs (page 9). Content of Vitamin A alcohol was obtained by difference.

Experimental Results

Table 9 presents the data on the total and esterfied vitamin A content of the livers and residual yolk sacs from 18- and 21-day old embryos.

The data indicates that 84.9 percent and 99.4 percent of the vitamin A in the residual yolk sac is the ester form after 18 and 21 days, respectively, of embryonic development. The livers contained 84.8 percent and 94.3 percent vitamin A esters after 18 and 21 days, respectively, of embryonic development.

Table 10 presents the data on the total and esterfied vitamin A content of infertile eggs incubated for 18 and 21 days.

These data show that there occurs a ten percent conversion of vitamin A alcohol to the ester form during the last three days of incubation, as the percentage of vitamin A esters increases from 20.4 to 31.8 percent.

Discussion

The percent of vitamin A ester in incubated, infertile eggs is not essentially different from that reported by Neff (39) for fresh eggs. Further studies of the apparent tendency of the vitamin A alcohol to convert to the ester form during the last three days of incubation should be made.

The vitamin A of the liver and residual yolk sac is predominantly in the ester form at 18 and 21 days of incubation. It appears from these data that the presence of the live embryo in some way effects the conversion of vitamin A alcohol to the

Table 9. The total and esterfied vitamin A content of the livers and yolk sacs of 18- and 21-day old embryos from eggs of hens receiving a ration supplemented with vitamin A at a level of 1,200,000 I.U. per 100 pounds of feed.

No. of hen from which eggs were obtained	Total vitamin A		Esterfied vit. A		Ester	
	yolk sac	liver	yolk sac	liver	yolk sac	liver
	micrograms				percent	
18 days incubation						
3974	50.0	----	41.2	----	82.0	----
	----	22.1	----	19.1	----	86.2
	48.2	----	38.1	----	80.2	----
	----	19.8	----	17.3	----	87.3
3989	----	----	----	----	----	----*
	----	19.4	----	13.6	----	70.0
9420	34.3	----	31.7	----	92.5	----
	----	11.8	----	11.3	----	95.8
MEAN	44.1	18.3	37.0	15.3	84.9	84.8
21 days incubation						
3974	38.8	----	41.4	----	107.	----
	----	19.7	----	18.1	----	92.8
9428	36.4	----	32.8	----	94.7	----
	----	17.3	----	16.8	----	97.0
3989	42.2	----	40.8	----	96.5	----
	----	20.7	----	19.3	----	93.0
MEAN	39.1	19.2	38.3	18.1	99.4	94.3

*Yolk sac lost during analysis.

Table 10. The total and ester vitamin A content of infertile eggs incubated for 18 and 21 days; eggs were from hens receiving the ration supplemented with vitamin A at the level of 1,200,000 I.U. per 100 pounds of feed.

No. of hen from which eggs were obtained	Total vit. A content per egg	Ester vit. A content per embryo	Vitamin A as ester in egg
	micrograms		percent
18 days incubation			
9428	88.6	17.3	20.2
	84.3	14.2	16.9
	89.3	16.1	18.0
	76.4	12.8	16.9
3974	108.0	25.5	23.4
	104.0	15.4	14.8
3989	101.0	28.4	28.2
	106.0	26.2	24.7
MEAN	94.5	19.5	20.4
21 days incubation			
9428	76.5	26.9	35.2
3974	107.0	34.2	31.9
	99.0	28.1	28.3
MEAN	94.5	29.7	31.8

ester, since the vitamin A of both the fresh egg and the incubated, infertile egg is predominantly in the alcohol form. Enzymatic action in the yolk sac of the fertile egg might account for this.

A further and more complete investigation of this problem is desirable.

SUMMARY

The vitamin A content of chick embryos, after 6, 12, 18 and 21 days of incubation, was determined. The embryos were from eggs of hens receiving rations supplemented with vitamin A at three different levels, (150,000 I.U., 300,000 I.U., and 1,200,000 I.U. per hundred pounds of feed). The vitamin A content of the embryos from the hens on the highest level of vitamin supplementation increased logarithmically with increase in embryonic age. The logarithmic relationship was not found after the eighteenth day of incubation and a smaller rate of transfer was evident when the maternal hen received feed supplemented with 300,000 I.U. of vitamin A per hundred pounds. The logarithmic relationship ceased to exist after the twelfth day of incubation of eggs from the hens receiving feed supplemented with vitamin A at a level of 150,000 I.U. per hundred pounds.

The changes in the vitamin A content of residual yolk sac of fertile eggs and of whole infertile eggs also were obtained. The loss in vitamin A content during incubation by both fertile and infertile eggs did not appear to be different.

The vitamin A content of the livers of 18- and 21-day old embryos was determined. The liver was the principal storage depot of vitamin A in these chick embryos.

The state of vitamin A, was determined, in the liver and residual yolk sac, of 18- and 21-day old embryos, and in infertile eggs incubated for 18 and 21 days. The vitamin A in the liver of the 18- and 21-day old embryos was predominantly in

the ester form. The vitamin A in the incubated, infertile eggs after 18 and 21 days of incubation was predominantly in the alcohol form.

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