Vitamin A Economy of the Developing Chick Embryo and of the Freshly Hatched Chick

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1. The changes in the net amounts of retinol, retinyl esters and retinal in both the developing chick embryo and the newly hatched chick were investigated. The embryo requires about 68 nmol of the vitamin for its growth, whereas the baby chick requires about 108 nmol during the first 7 days after hatching. 2. Retinal was present in the egg in fairly high concentrations at the beginning of the incubation but it virtually disappeared from the extra-embryonic tissue after day 17 of incubation. It was not found in the liver of the embryo or of the newly hatched chick up until day 7.

Chickens are a rather peculiar species of animals with respect to the metabolism of vitamin A: although male and female rats raised on a vitamin A-deficient diet supplemented with retinoic acid lose their ability to reproduce (Juneja *et al.*, 1964; Thompson *et al.*, 1964), similarly treated chickens do not do so; the males prove fertile and the females regularly lay eggs (Thompson *et al.*, 1965). However, if such eggs are fertilized the embryos fail to develop after incubation for 48 h (Thompson *et al.*, 1965) and injection of retinol derivatives into the eggs leads to the restoration of normal growth of the embryo (Thompson *et al.*, 1969). These observations have clearly indicated an absolute requirement of retinol and its derivatives for the normal development of the embryo.

Freshly laid eggs contain an amount of vitamin A in the form of retinol, retinyl esters and retinal (Neff *et al.*, 1949; Plack, 1960, 1963). This should naturally be expected to be sufficient for meeting the needs for the development of the embryo, because during incubation the embryo does not have access to external supplies of the vitamin. However, there has been no work to attempt a quantitative assessment of the actual requirements for vitamin A for the growth of the embryo and of the newly hatched chicks. Attempts were made in the present work to examine how far the amount of vitamin A usually present in the eggs is sufficient for meeting the needs for the normal development of the embryo and for the growth of the baby chick until external vitamin A becomes available.

Materials and Methods

The sources of the materials used have been described earlier (Mahadevan *et al.*, 1959, 1961; Ganguly *et al.*, 1959), except that neutral alumina for

chromatography was obtained from M. Woelm, Eschwege, Germany.

Fertile and non-fertile eggs from White Leghorn chickens were obtained from the Regional Poultry Farm, Hesarghatta, Bangalore. The eggs were incubated at 37°C and the humidity was maintained between 60 and 65% until day 18, after which it was raised to 80% until the day of hatching, and the eggs were turned twice a day. The required number of eggs were removed from the incubator on the given days and were weighed. After the egg was opened, the embryo was carefully separated from the extra-embryonic tissues, blotted on a filter paper and weighed to ascertain the age of the embryo. After hatching the birds were maintained for 7 days on a vitamin A-deficient diet of the following composition: casein, 12%; cracked wheat, 80.89%; dried yeast, 3%; NaCl, 0.5%; $\text{Ca}_3(\text{PO}_4)_2$, 1.5%; MnSO_4 , 0.002%; groundnut oil, 2%; riboflavin, 5 mg/kg of diet; vitamin D, 2000 i.u./kg of diet. At the given days the birds were killed by cardiac puncture while under ether anaesthesia and the livers and vitelline sacs were removed.

Since the egg white does not contain any vitamin A, no attempts were made to separate the yolk from the egg white and the whole extra-embryonic tissue, containing the yolk as well as the white, was taken for analysis. The livers of the embryos were too small, and therefore to obtain sufficient materials for analysis the livers of five embryos were pooled, but the yolk contents of each egg were analysed separately. The vitelline sacs and livers of the birds were analysed separately on each day until day 7. Since the size of the vitelline sac became smaller with time after hatching, the sacs from the following numbers of birds were pooled: for the first 2 days, two birds; for days 3 and 4, three birds; and for the subsequent 3 days, four birds.

The vitamin A from the extra-embryonic tissue was extracted by a modification of the method of Thompson *et al.* (1949) as follows: to the extra-embryonic tissue (yolk sac) about 120ml of light petroleum (b.p. 40–60°C) was added and the mixture was homogenized in a Waring Blendor for 20–30s. Ethanol (50ml) and water (10ml) were then added, the mixture was again homogenized for 10–20s and, after a pause for a few seconds, this was continued for another 2min. (We have consistently noticed that this procedure prevents the formation of emulsion.) The mixture was then separated in a separating funnel into an upper organic phase and a lower aqueous phase, and the lower aqueous phase was then extracted twice with light petroleum, 120ml being used each time. The three petroleum extracts were combined, dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was then dissolved in light petroleum and made up to a known volume in a volumetric flask.

However, the vitamin A from the embryonic tissues and from the livers was extracted by the method of Thompson *et al.* (1949).

Retinol, retinal and retinyl esters were separated on columns (8.0cm $\times$ 1.5cm) of 10%-deactivated Al_2O_3 . Retinyl esters were eluted with 1–2% (v/v) benzene in light petroleum, retinal was eluted with 1–2% (v/v) acetone in light petroleum, and retinol was removed with 4–5% (v/v) acetone in light petroleum. The retinol fraction obtained was free from lutein and other carotenoids.

Retinol and retinyl esters were determined by the Carr–Price reaction as described by Mahadevan *et al.* (1959), and retinal was determined by the thiobarbituric acid method described by Futterman & Saslaw (1961).

Results

Changes in vitamin A content in the vitelline sac and liver of the developing chick embryo and of the hatched chick

Vitelline sac. The changes in the content of total vitamin A and of its three forms, namely retinol, retinyl esters and retinal, in the vitelline sac from day 1 (day of setting) to day 21 (day of hatching), and then up until the 7th day after hatching are presented in Fig. 1(a). The average total vitamin A content was about 253nmol on day 1 and 101nmol on the day of hatching. On the day of the beginning of incubation the proportions of retinol, retinyl esters and retinal were 4:1:1 but on the day of hatching they were 1:5:0. The retinol content of the vitelline sac showed a steady but slow decrease until day 9, after which it declined more rapidly so that on day 21 only about 16nmol was present. The content of retinyl esters did not show any increase until day 7, after which it increased slowly until day 15 and on day 17 a sharp

increase was noticed; thereafter it decreased again. The content of retinyl esters on day 21 was twice that originally present on day 1. The retinal content, on the other hand, gradually decreased and it could not be detected after day 17.

The results (Fig. 1a) of the analysis of the vitelline sacs of the newly hatched chicks maintained on the vitamin A-deficient diet show that retinol content fell sharply from 16nmol per vitelline sac to 4nmol during the first 24h, and thereafter did not change much. The content of esters, on the other hand, decreased rather markedly as long as the birds were kept on this diet. It was interesting that, although the decreases in the total vitamin A content and in the weight of the vitelline sac (7.88g down to 0.20g) ran parallel, the decrease in the retinol content during the first day after hatching was almost twice that in the weight of vitelline sac. No retinal could be detected in the vitelline sacs of the hatched chicks.

Liver. Since the amounts of liver were too small for analysis at the earlier stages of incubation, retinol and retinyl esters could be measured in the embryonic liver only from day 9 onwards. Fig. 1(b) shows that retinol could be found in the embryonic liver only after day 15 and its content per liver did not increase significantly during the rest of the incubation period and after hatching. In contrast, retinyl esters of the embryonic liver increased from 0.68 nmol per liver on day 9 to 31.50nmol per liver on day 21; obviously the increase in the total vitamin A content of the liver was entirely accounted for by the esters. The total vitamin A and retinyl esters of the livers of the hatched chicks decreased steadily until day 4, and then remained more or less steady. Although significant amounts of retinol and retinyl esters were present, retinal could not be detected in the livers of the embryo and of the hatched chicks.

Utilization of vitamin A by the embryo during incubation and by the hatched chick

The results summarized in Table 1 show that the egg yolk contained an average of 253nmol of vitamin A on day 1 of incubation. However, the control values obtained from simultaneous incubation of non-fertile eggs for 21 days showed that there was a net loss of an average of 50nmol of the vitamin, presumably owing to destruction. Therefore from about 203nmol of total vitamin A available during the incubation of fertile eggs, 135nmol could be recovered in the vitelline sac plus liver on the day of hatching. This means that the embryo uses 68nmol of the vitamin for its growth during the 21 days of incubation. However, at day 7 after hatching (on the vitamin A-deficient diet) only 27nmol of vitamin A was found in the vitelline sac plus liver. It is therefore clear that the bird uses about 108nmol of the vitamin for its growth for the 7 days after hatching.

Fig. 1. Changes in vitamin A in (a) vitelline sac, and (b) liver of developing chick embryo and hatched chick

Two sets of independent experiments were done with a minimum of ten eggs or ten birds on each day of the experiment. The values reported here are averages of 20 points. The extraction, separation and determination of vitamin A are as described in the Materials and Methods section. The vertical bars represent the s.d. ○, Total vitamin A; ●, retinol; △, retinyl esters; ▲, retinal. The arrow indicates time of hatching.

Discussion

The main aim of this work was to examine the vitamin A economy of the developing chick embryo. The chicken lays the egg containing all materials necessary for meeting the nutritional requirements of the embryo during its development and also of the

newly hatched bird during the first few days of its life; vitamin A is one such essential nutrient.

It has been shown here that retinol is the major form in which vitamin A is deposited in the freshly laid eggs. This is in agreement with the findings of earlier workers (Neff *et al.*, 1949; Plack, 1963). During the

Table 1. *Summary of the changes in the different forms of retinol during incubation of eggs and after hatching of chicks*

The procedures used are as described in Fig. 1. The values are expressed as nmol of the vitamin per vitelline sac or liver.

Form of vitamin A	Fertile eggs					Yolk of non-fertile egg	
	Day of setting	Day of hatching		7th day after hatching		Day of laying	After 21 days of incubation
		Vitelline sac	Liver	Vitelline sac	Liver		
Retinol	172	16	2	2	2	165	128
Retinyl esters	41	85	32	5	18	38	30
Retinal	40	0	0	0	0	35	30
Total	253	101	34	7	20	238	188

incubation the retinol and total vitamin A contents of the extra-embryonic tissue steadily decreased until day 15, after which there was a steep rise in the retinyl esters that was accompanied by a sharp decrease in the retinol content. However, from day 17 onwards the retinol content and that of its esters steadily decreased. Parrish *et al.* (1951) also reported an increase in the retinyl ester content of vitelline sac at later stages of incubation.

Although very small amounts of retinyl esters began to appear in the liver on day 9 and continued to increase steadily until the day of hatching no retinol could be detected in the liver until day 15 and its content remained almost constant till day 7 after hatching. It is possible that retinyl esters are synthesized in the vitelline-sac membrane and are directly transported to the liver for storage. It is also possible that the retinyl esters synthesized in the vitelline sac are transported to the intestine, where they are hydrolysed, after which retinol is re-esterified there and stored in the liver.

The results of analysis for retinal have shown that although the fresh egg contains considerable amounts of retinal its concentration slowly and steadily decreases, so that from day 19 onwards it could be detected neither in the extra-embryonic tissue nor in the liver of the embryo and of the bird. Plack (1963) had measured the retinal contents of the yolk of hen's egg at day 1 and day 21 of incubation and had found a decrease of 84% of the initial retinal values. The disappearance of retinal from the extra-embryonic tissue is rather interesting, because Plack *et al.* (1966) had observed a 10-fold increase in the retinal concentration in the blood of hens at the time of egg laying. Other workers also have demonstrated the presence of significant amounts of the aldehyde in hen's egg (Plack, 1960; Plack & Kon, 1961; Winterstein & Hegedüs, 1960), in several marine fish (Plack *et al.*, 1959), and in the eggs of frogs and whiting (Pollard & Bieri, 1959). These observations

had led to the natural speculation that retinal might be involved in the reproductive processes of animals. But Plack *et al.* (1964) suggested that retinal in hen's eggs is of no apparent significance for the development of the chick embryo. The present results should fully support such an idea that retinal is not required for the development of the chick embryo (at least at the later stages), nor for the newly hatched chicks.

The vitelline sac that is taken inside the body cavity by the baby chick at the time of hatching contained about 101 nmol of the total vitamin A (about 253 nmol) originally present in the egg; this is three times the amount found in the liver. The content of both retinol and retinyl esters of the baby chicks maintained on the vitamin A-deficient diet decreased with time in the vitelline sac as well as in the liver, and over the period of 7 days the decrease was more pronounced in the vitelline sac. These results agree with the findings of Gažo *et al.* (1967), except that they observed an increase in the ester content during the first 24 h in the vitelline sac as well as in the liver, but we have noted a decrease. One could probably conclude from the present results that during the early part of its life the baby chick uses the vitamin A of the vitelline sac for its needs.

It can be calculated from these results that the embryo uses about 68 nmol of vitamin A for its growth during the 21 days of incubation, and the bird uses about 108 nmol during the first 7 days of its life. Therefore the initial supply of about 253 nmol of the vitamin in the freshly laid egg should be sufficient for meeting the requirements of the bird until it becomes independent and is able to take in external vitamin A.

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