Iron Metabolism and Haemoglobin Formation in the Embryonated Hen Egg

1. THE OVERALL PRODUCTION OF HAEM PIGMENTS DURING INCUBATION

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Early work on the iron content of hen eggs was reviewed at some length by Needham (1931). The results quoted, however, are very much at variance with one another, and should probably be ignored altogether, because of the inadequacy of the analytical techniques then available. The later work, briefly reviewed by Cruickshank (1941), gives the impression, fully supported by analyses done during the present experiment, that the egg contains on the average about 1 mg. iron, but that there is a large variation between individual eggs. It is agreed that this iron is present almost entirely in the yolk. An observation, first made by Hill (1931), and confirmed by all later workers, showed that practically the whole of the iron present in the unincubated egg reacts with 2:2'-dipyridyl in the presence of a reducing agent to give a pink colour. Since the iron of haem does not give this reaction, it is evident that haem pigments are virtually absent.

It is obvious from the appearance of partially incubated eggs, and from a most superficial consideration of the young chick, that during the 21 days of incubation a large part of the yolk iron is incorporated, presumably after transference to the embryo, into haemoglobin and related pigments. Surprisingly few studies of this aspect of embryo metabolism seem to have been made, and those which have been published are incomplete.

Sendju (1927) washed the blood out of the vessels of 7-, 14- and 19-day embryos and determined the Hb as acid haematin with a Sahli haemoglobinometer. Schönheyder (1938), in the course of an investigation of coproporphyrin metabolism, determined Hb in the 'embryonic areas' of incubated eggs almost daily up to 10 days, and again in 2-day-old chicks which he bled to death. He used the admittedly approximate pyridine-haemochromogen technique of Anson & Mirsky (1929) for his Hb measurements in the embryo. This technique can be expected to determine myoglobin and perhaps other pigments as well as Hb. The data are so scanty that a comparison is scarcely worthwhile, but it is surprising, and by no means explicable on the basis of the perhaps less specific method used by the later worker, that Sendju's figure (23.7 mg.) for Hb in 14-day embryos is very little greater than Schönheyder's (21 mg.) at 10 days. The work described in 'his paper suggests that Sendju's 14-day figure is many times too low. Neither of these workers made any Fe determinations, and it is not quite clear whether either included the membranes in the material

analysed. This is a point of no small importance, since both yolk sac and chorio-allantoic membrane are richly endowed with blood vessels, and, until the development of the chick is nearly complete, they form a very substantial proportion of the total amount of living matter in the egg.

The accumulation of Fe in the body of the developing chick embryo has been fairly fully studied by McFarlane & Milne (1934), but they did not include the yolk sac (and probably other membranous material) in their analyses. They were aware of the error thus introduced, but the picture they gave is incomplete in other respects, since no determinations were made at any stage of the Fe remaining in the yolk. In the liver, but not in the whole chick, they distinguished between total and non-haem Fe, for which they used the method of Hill (1931). These workers were thus able to show a marked accumulation of Fe in the embryonic liver towards the end of incubation. They seem to have found that on the twenty-first day there was about 0.2 mg. non-haem Fe in the liver, which is about one-sixth of the total amount of Fe present in the entire chick. This figure (0.2 mg.) is 10 times greater than that calculated from the authors' figure for total liver Fe, but it is evident from the rest of their analyses and calculations that the liver Fe values which they quote are 10 times too small.

The present work aims at filling some of the gaps in our knowledge of iron metabolism in the embryo. As no direct method for the determination of haemoglobin has yet been found to be reliable when applied to homogenates of whole eggs, determinations have been made, at all stages of incubation, of that proportion of the total egg iron which does not react with 2:2'-dipyridyl in the presence of a reducing agent. Attempts have been made to interpret the results in terms of haem synthesis and haemoglobin production. In this preliminary survey the body of the embryo chick was not separated from its membranes or from the rest of the egg.

EXPERIMENTAL

General. All the eggs used during these experiments were supplied by the Poultry Research Centre, Edinburgh, from their flock of Brown Leghorn hens. The eggs were incubated for various periods under the usual conditions of temperature, humidity, etc. Eggs taken after incubation for 8 days or longer were immersed in acetone-CO₂ for 5 min., stripped of their shells, and homogenized in a glass homogenizer (Potter & Elvehjem, 1936). Eggs incubated for periods less than 8 days burst when immersed in acetone-CO₂. These were therefore frozen in powdered CO₂. The solidified egg was

broken up roughly with a nickel spatula and the still frozen pieces homogenized without the addition of any liquid. For eggs incubated for 18 days or more an equal volume of ice-cold water was added and a preliminary treatment for about 30 sec. in a blender of the Waring type was used. Careful blender tests have shown that no iron is introduced into the homogenate from the steel knives of the blender. The mixture from the blender was refrozen before glass homogenization. After about the sixteenth day small amounts of feathers and connective tissue form knots resistant to the most vigorous treatment in the glass homogenizer. Such material was discarded. Even in eggs on the point of hatching, it does not amount to more than about 1 g./egg. Sample portions of homogenates prepared in this way were analysed by the techniques described below, and the results obtained used to calculate the amount of Fe (total or non-haem) present in the whole of each individual egg. Each chemical analysis was done in triplicate.

Total Fe. This was determined by a modification of the method previously used for blood (Ramsay, 1944, 1946). The major change was necessitated by the richness of eggs in ether-soluble substances, a fact which makes the homogenate very difficult to digest, except with a very large excess of $\text{H}_2\text{SO}_4\text{-HClO}_4\text{-HNO}_3$ mixture. Even though digestion appeared complete, unsatisfactory end points were frequently observed in the final titration. The following technique, of which the unusual feature is the extraction of fatty material with ether before the wet digestion, was therefore adopted. A great economy in the digestion acids, with a consequent reduction in the unavoidable blank is thereby effected, and the end point difficulty seems to have been almost entirely surmounted. Homogenate (1 ml.) is pipetted into the usual small flask, followed by 1 ml. of water and 1 ml. of conc. H_2SO_4 (A.R.). A glass bead is dropped in and the mixture is heated carefully over a small flame until it is apparently homogeneous. The flask is then immersed in boiling water for 45–60 min., during which time a thick, yellow, oily layer of fatty acids separates and floats. After cooling, this layer is removed by extraction successively with 2×3 ml. and 2 ml. of redistilled ether. The ether is removed after each extraction with a very finely tipped Pasteur pipette. The flask is returned to the water bath to evaporate the last traces of ether and after about 20 min. 0.6 ml. of glass-distilled HNO_3 and 0.5 ml. 60% (w/v) HClO_4 (A.R.) are added slowly and very cautiously. The digestion and titration may now be completed as described in the earlier papers, but it is an advantage to leave the flasks in the boiling water bath for about 1 hr. before heating over a free flame. A micrometer syringe burette may be used for the final titanous sulphate titration. It should be noted that only the low concentration of Cu in the egg as a whole (Fe : Cu $\approx$ 100 : 1, calculated from figures given by McCance & Widdowson, 1940) makes it possible to use the simple direct Ti titration as a measure of Fe.

2:2'-Dipyridyl Fe. Hill (1931) added to egg yolk dilute acetate buffer, solid dipyridyl and solid $\text{Na}_2\text{S}_2\text{O}_4$, afterwards making the mixture up to $5 \times$ its original volume with ethanol. In the present work it was found that the colour produced with some commercial specimens of $\text{Na}_2\text{S}_2\text{O}_4$ is not stable, and that the error cannot be corrected by the addition of more $\text{Na}_2\text{S}_2\text{O}_4$. Hydroxylamine was therefore employed as reducing agent, in accordance with a recommendation of Jones (1948). Sherman, Elvejhem & Hart (1934) observed that with certain animal tissues full recoveries of Fe could not be attained without using acid of considerably greater

concentration than is present in 0.1N-acetate buffer. Their explanation was that the usual buffer failed to maintain a sufficiently acid reaction. Although this seemed surprising since Hill obtained full colour development up to pH 8, it was thought wise to use an exceptionally concentrated acetate buffer at a pH of about 4.5. The high salt concentration has the additional advantage of reducing to a minimum the volume of ethanol required to produce a clear solution for the final colorimetric measurement. It may be mentioned that the use of $(\text{NH}_4)_2\text{SO}_4$ to aid the production of a clear filtrate results in the retention of a high proportion of the ferrous dipyridyl complex with the precipitate. No such effect has been noticed with the acetate concentration finally adopted.

The procedure for all the non-haem Fe analyses quoted here is as follows: 2 ml. egg homogenate is measured into a test tube graduated at 16 ml. To this is added 5 ml. of a solution which is 2M with respect to Na acetate, 3M with respect to acetic acid, and which also contains 0.1% redistilled 2:2'-dipyridyl and 0.5% hydroxylamine (in terms of the hydrochloride). The last substance must be virtually freed from Fe by three recrystallizations from the strong acetate buffer containing traces of dipyridyl. After careful mixing, the tube is heated for 10 min. in a briskly boiling water bath, and allowed to cool to about 50°. It is then made up to the mark with ethanol, vigorously shaken, and allowed to cool to room temperature. After a final volume adjustment with ethanol, the mixture is filtered through a Whatman no. 42 paper, and the pink colour is measured in a photo-electric colorimeter, using a green filter with a rather narrow transmission band (Ilford 604 or 624). It is necessary to carry out a blank analysis with a duplicate sample of homogenate, which is put through the whole procedure with a buffer solution free from dipyridyl. All eggs give a yellow colour in the blank estimation, but up to 12 days there is negligible absorption of the light transmitted by either of the above filters. After the twelfth day, however, the blank colorimeter reading gradually increases until at the end of incubation it may be nearly half the total. There is no definite evidence concerning the nature of the substance responsible for the blank reaction; the fact that it absorbs in the green and appears in increasing amount as the Hb rises suggests that the substance may be a haem derivative, but, on the other hand, it first develops at the same time as the down, which in these Brown Leghorn chicks is very highly pigmented. The two Fe methods have been standardized throughout against the same solution of ferric ammonium sulphate.

The difference between total and dipyridyl iron has been assumed to be iron in combination with protoporphyrin IX or some closely related stable substance, and is described here as haem iron. There is a large body of evidence to justify this assumption. On the one hand, it is well known that all but the most stable covalent compounds of iron can be made to react with 2:2'-dipyridyl with the aid, for ferric compounds, of a reducing agent; this has been demonstrated very convincingly by Jones (1948). Although he chose *o*-phenanthroline as his colour-developing agent, it seems from his paper that closely similar results are obtained with dipyridyl. It was not necessary to make any further check on this aspect of the test, but since most of the older

dipyridyl techniques do not involve heating, it has been thought wise to prove the non-reactivity of haem iron. When a preparation of haemin containing 8.42% iron (determined by titanium titration after wet digestion) was put through the dipyridyl procedure described above, only 0.18% of the total iron was found to react. This result is conclusive, as far as haem is concerned, and the reasonably close agreement between the two methods, when both are applied to unincubated eggs, confirms the absence from such material of any other iron compounds which might not react with dipyridyl.

RESULTS AND DISCUSSION

The two analytical techniques described above have been applied to homogenates prepared from a total of 115 eggs incubated for various periods between

Fig. 1. The total iron content of 115 Brown Leghorn eggs.

0 and 21 days, and before discussing the results in detail it seems desirable to emphasize the considerable variations which have been found in the iron content of the eggs. Since unpublished observations in this laboratory show variations of similar magnitude in other egg constituents, caution should be exercised in the interpretation of all experiments based on small numbers of eggs. The iron content of the eggs used in the present work averaged 1.067 mg. per egg, with a standard deviation of ± 0.20 mg. and an extreme range of 0.64–1.72 mg. per egg. The values were approximately normally distributed, as may be seen from Fig. 1. No relation between the size of the egg and its iron content was noticed, as very large eggs frequently have quite low iron concentrations and vice versa; but individual variation is so great that it might be necessary to analyse some hundreds of eggs before definite conclusions could be drawn.

Twelve unincubated eggs were analysed for both total and non-haem iron. The proportion of non-haem iron ranged from 95 to 101% of the total, with a mean of 98% (S.D. ± 1.5 %). These results are in close agreement with those of previous workers,

e.g. Hill (1931), and justify broadly the analytical techniques. The present procedure is, however, quite inadequate for detecting, let alone determining, the small but definite quantity of haem which is synthesized during the early days of incubation; already at the third day blood can be seen in the vessels of the yolk sac with the naked eye, but not until about the sixth day is the analytical difference between total and non-haem iron significant. By about the tenth day the difference probably provides a fairly accurate estimate of the amount of haem iron present.

Fig. 2. The increase of haem iron during the incubation of eggs.

Fig. 2 shows the progress of the haem iron content, in mg. per egg, throughout the course of incubation. All the points on the figure, except that for the twenty-first day, represent the means for four to eight eggs. The last point represents only two eggs; this is chiefly because it is very difficult to make eggs, on the point of hatching, into a true homogenate, without running the risk of introducing extra iron. These two eggs, however, were both well chipped at the moment they were frozen, and had thus reached the same stage of development. Moreover, the results agree closely with one another, and as the point fits in so neatly with the others there seems to be no reason to doubt its validity. This justification applies more particularly to the calculations of haem iron as a percentage of the total iron which are shown in Fig. 3. It will be seen from Fig. 2 that haem synthesis proceeds comparatively slowly until the eighth day, when the egg contains about 0.08 mg. haem iron, and the curve then steepens markedly, so that at 10 days the value for haem iron is about 0.15 mg., at 14 days 0.42 mg., at 18 days 0.76 mg. and at hatching 1.03 mg.

The mean haem iron content of the later eggs exceeds considerably the total iron content of some of the individual eggs used in the investigation. This observation has prompted the construction of Fig. 3, showing the proportion of the total iron present at any given stage of incubation as haem iron. From the twelfth day onwards this proportion was remarkably constant for eggs of equal incubation

Fig. 3. The increase of haem iron, expressed as a percentage of the total iron, during the incubation of eggs.

periods, and the whole figure throws a new light on the matter. Thus, between the sixth and the tenth day about 2% of the total iron is incorporated into haem each day. From the tenth day at least until the sixteenth day the rate of synthesis expressed in this way is nearly constant, but about three times as great: something over 6% of the total being transformed into haem iron each day. During the last 5 days there is a slight fall in the rate, but this may be more apparent than real. There is at this stage some formation of bile pigments which probably means that some breakdown of haem is taking place; the quantities of haem observed in the present experiments must be, of course, the resultants of synthetic and destructive processes.

The fact that during the last 8 or 9 days of incubation the haem iron forms a relatively constant proportion of the total is worthy of further comment. This applies to all the later groups of eggs, but a series of 18-day eggs with a particularly wide range of total iron contents illustrates the point particularly well. In Fig. 4 the haem iron is plotted against the total iron for each of the eight 18-day eggs. It is seen that the amount of haem increases with the total iron, and that the points for the individual eggs lie quite close to the straight line calculated from Fig. 3, using the equation: $\text{Haem-Fe} = 0.59 \times \text{total Fe}$. Thus in the embryonated egg, as in so many other biological systems, the quantity of iron available may be one of the limiting factors in haem synthesis. Whether it has an appreciable influence on the

development of the embryo or the hatchability of the egg is at present uncertain. The following observation is, however, at least suggestive: of the 115 eggs used for these experiments 37, or 32.2%, contained 0.95 mg. iron or less, while of the thirty-eight eggs out of the total which were incubated for 14 days or longer only four, or 10.5%, were low in iron to this extent. These figures would be consistent with the hypothesis that embryos in eggs particularly low in

Fig. 4. The relation of haem iron to total iron in eggs incubated for 18 days.

iron tend to die during the first fortnight of incubation. The analysis of unsuccessfully incubated eggs has not yet been undertaken, but may provide an answer to this important question.

Comparison of the amount of haem pigment in the egg with the total weight of the embryo

No observations on embryo weight were made during the present investigation, and the technical skill required for making reliable measurements of the weight of the membranes was not available, but figures based on more than 2000 eggs are given by Byerly (1932). As the small number of embryo weights recorded in the present work agree quite well with those of Byerly, it was considered safe to use his figures, especially as the comparisons involve not absolute weight but the proportion of the final weight reached at different stages in incubation. In Fig. 5 the period of incubation, from the tenth until the nineteenth day, is plotted separately against the haem iron, the embryo weight excluding membranes, and the embryo weight including the membranes, each calculated as a percentage of its magnitude at hatching. The haem values have been calculated on the basis of the analytical observation that at 21 days 72% of the total iron in the egg is present as haem. Although it is true that a larger number of eggs might have given a mean percentage slightly different from this, the general trend in Fig. 5 would not have been altered. The embryo and membrane figures have been calculated from Byerly dry weights. As the body of the chick becomes progressively 'less wet' during the latter half of incubation, somewhat

different figures are obtained if wet weights are used, but again the general trend of the results is the same. Comparison of the graphs shows that a much larger proportion of the final amount of haem than of the gross body weight has been manufactured during the earlier stages of incubation. Thus, at 12 days, 37 % of the final amount of haem has been accumulated, whereas the body of the chick has only reached 10 % of its final weight. At 15 days about 63 % of the final amount of haem has made its appearance, but the chick-body weight is still only 40 % of its final value. By 18 days the two values

Fig. 5. The relation of haem synthesis to chick embryo growth. ●—●, haem content as a percentage of its final value; □—□, embryo dry weight as a percentage of its final value (Byerly, 1932); ○—○, embryo + membranes, dry weight, as a percentage of their final value. All these functions are plotted against the period of incubation.

are respectively 81 and 74 %, while a day later they are even closer at 87 and 83 %. At 21 days the values must of course both be 100 %; what requires emphasis is that at certain stages of development the rate of haem synthesis and hence almost certainly the rate of haemoglobin manufacture is relatively much higher than that of gross growth. The most probable explanation is suggested by the appearance of the egg, with its extensive yolk sac and allantoic membrane, both obviously richly supplied with blood: except towards the end of incubation, when these membranes disappear, a large part of the egg haemoglobin cannot be in the body of the chick at all, but must be circulating in the membranes. That this is at least partially true is shown by the middle curve in Fig. 5, which describes the change in embryo weight including the membranes, again calculated at each stage as a percentage of the final value. This line lies a good deal closer to the haem graph than does that for embryo weight alone, and the difference that still remains may well be largely accounted for

by the fact that the membranes are organs of a rather uncommon shape, with a blood supply which, relative to their bulk, is unusually copious. The foetal placenta may perhaps impose similar and rather high demands on the haem-synthesizing mechanism at certain stages of the development of the mammalian embryo.

Although all attempts to make direct haemoglobin determinations on egg homogenates with a precision at all approaching that of these iron methods have so far been unsuccessful, it is possible to calculate approximate haemoglobin figures from the haem iron estimations. Drabkin (1948) has published figures for the total content in several mammalian species of the three chief haem compounds: haemoglobin, myoglobin and cytochrome c. In the adult rat haemoglobin amounted to 92 % of the total, in man 96 %, and in the cow 88 %. In the horse the proportion was somewhat lower, only 75 %, chiefly because of the presence of an unusually large quantity of myoglobin. Although a comparison between adult mammals and embryo chicks is somewhat hazardous, it seems likely that no great error will result from the assumption that 90 % of the haem in the egg is present in haemoglobin. Such a figure might be too high in the earliest stages of development, but these are not considered here, and might again be too high towards hatching time, since no information is available concerning the amount of myoglobin in the muscles of the unhatched chick. The error is unlikely to be greater than that involved in rinsing from embryo or chick vessels a fluid which clots so very readily as chicken blood. Table 1 summarizes haemoglobin figures from the papers of

Table 1. *Haemoglobin content of eggs and embryos*

(mg. Hb/egg or/embryo)			
Age (days)	Embryos (Sendju, 1927)	Embryonic areas (Schönheyder, 1938)	Whole eggs (This work)
6	—	4.3	—
7	7.6	—	—
8	—	15.7	26
10	—	21	43
14	23.7	—	110
19	141	—	185
21	—	—	208
2-day-old chicks	—	173	—

Sendju (1927) and Schönheyder (1938), and from the present work, calculated on the assumption which has just been discussed and the additional one that embryonic chick haemoglobin, like that from so many other sources, contains about 0.335 % iron. The calculations have been made from the values in Fig. 3 for the proportion of the total iron present as haem iron, and from the general mean value of

1.07 mg. total iron per egg. The present estimates are at all times higher, sometimes very much higher, than the previous ones, probably because of the inclusion of all the haemoglobin-containing material in the egg. Schönheyder believed that his figure for the total haemoglobin content of 2-day-old chicks might be low, and if that is taken into account his value is in good agreement with the present one. A rough estimate of the haemoglobin content of the hatching chick is about 200 mg., although of course it is clear from Fig. 4 that there must be large variations among different individuals.

SUMMARY

1. A modification of the wet-digestion titanium-technique for total iron, suitable for application to eggs, is described. Total iron in 115 eggs has been found to average 1.07 mg. per egg.

2. A modification of the 2:2'-dipyridyl method for the determination of non-haem iron is described.

It is confirmed that almost the whole of the iron in unincubated hen eggs is non-haem iron.

3. The increase in haem iron during the course of incubation is described. It amounted to 0.14 mg. per egg after 10 days' incubation, 0.41 mg. at 14 days, and 1 mg. at 21 days.

4. During the last 9 days of incubation a fairly constant proportion of the total iron is present as haem iron at any given stage of development. This increases from 40% at 14 days to 72% at 21 days.

5. Haem synthesis is discussed in relation to: (i) the total amount of iron present, and (ii) the growth of the embryo and its membranes.

6. Approximate haemoglobin values are calculated from the haem iron figures. It is concluded that the average egg on the point of hatching probably contains about 200 mg. haemoglobin.

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Studies on Pituitary Adrenocorticotrophin

1. ULTRAFILTRATION OF THE HORMONE

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Smith (1926) demonstrated that the adrenals of the rat undergo atrophy following hypophysectomy. He also showed (Smith, 1930) that the anterior lobe of the pituitary gland contained a factor which could bring about the reversal of this adrenal atrophy. He further found that both atrophy and repair were limited to the adrenal cortex. The factor has since been termed the adrenocorticotrophic hormone. Later, several workers attempted to isolate and characterize the hormone (Collip, Anderson & Thompson, 1933; Collip, 1938; Lyons, 1937; Bates, Riddle & Miller, 1940). These investigations sug-

gested that the hormone was protein or polypeptide in nature, but differed from the other anterior pituitary hormones in its remarkable thermostability.

Sayers, White & Long (1943) and Li, Evans & Simpson (1943) simultaneously published details of the preparation, from pig and sheep pituitaries respectively, of proteins of high adrenocorticotrophic potency which appeared to be homogeneous by the criteria of sedimentation, electrophoresis and constant solubility. The preparations from pig and sheep glands, and also material prepared by a