2. It has been shown that the *S*-ethyl and *S*-phenyl isomers of parathion and the oxygen analogue of parathion have a powerful inhibitory action.

3. The variable inhibition produced by samples of parathion can be entirely ascribed to contamination

with the *S*-ethyl isomer of parathion, and a highly purified sample, containing only a trace of this isomer, has a very low inhibitory action.

We are indebted to Messrs Albright and Wilson Ltd., and to Messrs Plant Protection Ltd., for supplying samples of parathion and analogous materials.

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Iron Metabolism and Haemoglobin Formation in the Embryonated Hen Egg

2. SOME OBSERVATIONS ON THE EMBRYONIC BLOOD SUPPLY

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In a recent paper (Ramsay, 1950) some evidence was presented suggesting that the membranes of the developing egg contain a substantial proportion of the total quantity of haem iron in the egg, and hence of the haemoglobin and blood. This paper discusses in greater detail the changes which take place in the amount of haem iron and its distribution between the body of the embryo and its membranes during incubation.

EXPERIMENTAL

General

Eggs from Brown Leghorn hens weighing 50–60 g. were incubated for various periods and frozen as soon as possible after removal from the incubator in thoroughly prechilled acetone- CO_2 . The shell was removed, and the embryo body dissected from the remainder of the egg contents. In this way the separation was easily made without loss of blood, and it is believed that the freezing was sufficiently rapid to prevent any serious redistribution of blood taking place.

Homogenates prepared with the addition of suitable quantities of ice-cold water from the embryo and the 'rest' (the 'rest' comprised yolk, white, allantoic and amniotic fluids, and the membranes, but not the shell) were separately analysed for non-haem and total Fe by the methods described below. In the interpretation of the results it has been assumed that the haem Fe in the 'rest' was derived entirely from the haemoglobin of the blood circulating in the membranes. These constitute the only living tissue outside the body of the embryo, and they are such highly vascular structures that the cytochrome and other haem compounds of the membrane cells themselves must surely be even less

important quantitatively than in less vascular systems. Where necessary, the rather less justifiable assumption has been made that in the embryo body also the amount of non-haemoglobin haem Fe might be neglected. This is based on the work of Drabkin (1948), who has shown that in the bodies of several species cytochrome and myoglobin do not account for more than about 10% of the total haem.

Non-haem iron

Reagents. Dipyriddy reagent: a solution containing 2.5 M-acetate, pH 4.6, 2,2'-dipyridyl, 0.2%, NH_4OH , HCl, 0.5%. Ethanol, redistilled.

Procedure. Homogenate (1 ml.) was treated with 4 ml. dipyriddy reagent and the mixture heated for 5 min. in a boiling-water bath. After cooling, the contents of the tube were made up to 12 ml. with ethanol and filtered through a no. 42 paper when the precipitate had coagulated. The Unicam D.G. Spectrophotometer at a wavelength setting of 520 m μ . was found very suitable for measuring the density of the pink colour. 'Blanks' were done in the same way, but with acetate buffer alone instead of the complete reagent.

Total iron

The technique for estimation of total Fe was based on the belief that the procedure just described does in fact determine all non-haem Fe, and on the knowledge (Peters, 1947) that haem pigments can be decomposed by H_2O_2 . It has been found that the decomposition is not always effective at low temperatures, perhaps because catalytic destruction of H_2O_2 interferes with the desired reaction. Moreover, H_2O_2 interferes with the subsequent dipyriddy reaction, but excess can be easily destroyed with Na_2SO_3 .

Reagents. H_2O_2 , 3% (w/v) in 2 M-acetate, pH 4.6. $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$, 40% (w/v). Neither of these reagents should be more than a week old.

Dipyridyl reagent: this was exactly twice as concentrated, with respect to all the solutes, as the non-haem Fe reagent.

Ethanol, redistilled.

Procedure. Homogenate (1 ml.) was warmed to a temperature not lower than 50° and H_2O_2 (0.2 ml.) added. The mixture was shaken occasionally during 5 min., and again after the addition of 1 ml. Na_2SO_3 . After a further 5 min. dipyridyl reagent (2 ml.) was added, and the tube heated for 15 min. in a boiling-water bath. After cooling, the volume was made up to 12 ml. with ethanol and the estimation completed in the same way as for non-haem Fe.

The NH_4OH in the dipyridyl reagent is not strictly necessary in this procedure, as sufficient sulphite is always left after the destruction of excess peroxide to reduce all the Fe. It was added in order that as many features as possible might be common to the total and non-haem Fe methods. It cannot be replaced by sulphite in the latter technique, since the heating of haemin or haemoglobin solutions with sulphite and dipyridyl in weakly acid solution results in the rapid formation of the ferrous dipyridyl complex ion.

Both methods gave satisfactory recoveries of iron added to biological materials such as milk, egg yolk, or chick embryo homogenized at different stages of development. With the total Fe method the extra iron was added as oxyhaemoglobin. Possible inhibitory effects were investigated by going through the long list of substances tested by Jones (1948). Iron added as ferro- or ferri-cyanide did not react completely at pH 4.6, but these complexes are unlikely to be found in biological material. The curious apparent inhibitory effect of pyruvate observed by Jones was not found when sufficient NH_4OH was added to leave an excess after the formation of the oxime of pyruvic acid. Oxalate in the large amount of 200 mg. per tube interfered seriously with colour development, but it is considered very unlikely that in actual practice inhibitory concentrations of oxalate would be encountered.

It is well known that unincubated eggs contain no haem iron (Hill, 1931; Ramsay, 1950). In five such eggs analysed by these two methods the non-haem Fe ranged from 99 to 100.5% of the total Fe. In the embryos and 'rests' of partially incubated eggs total Fe values found by this technique agreed closely with those found by either the dipyridyl reaction or titanium titration after wet ashing, showing the absence from these materials of Fe complexes not decomposed by heating with dipyridyl and a reducing agent after preliminary treatment with H_2O_2 .

Homogenates prepared from the embryos and 'rests' of eggs incubated for 14 days (in these the 'rest' contains substantial quantities of haem Fe) were heated for periods up to 2 hr. with the non-haem Fe reagent. It was found that the pink colour developed rapidly for about 3 min. and remained constant for a further 15–20 min. After this, it increased slowly in intensity until at the end of 2 hr. it was nearly as intense as that obtained after decomposition of haem pigments with H_2O_2 . It was concluded that although haem pigments are slowly attacked by the non-haem Fe reagent at the boiling point, there is not, in these materials, significant decomposition in the 5 min. period adopted as a routine.

Although it was believed that the experiments described in the preceding paragraphs provided good circumstantial evidence in favour of the assumption that the difference between experimentally determined total and non-haem Fe is in fact haem Fe, it was felt that direct evidence would be preferable. Attempts were therefore made to compare the

methods of this work with the pyridine haemochromogen technique of Flink & Watson (1942), which was designed for application to urine, faeces and blood plasma. The results of the comparison were disappointing, apparently because of erratic pyridine haemochromogen determinations, but they did suggest that the fundamental assumption is not entirely unsound, at least for the present experimental material.

Caution, however, is required in the application of the methods to other materials. The non-haem Fe method gives erroneous results when applied to whole blood or to any other material containing an unusually large excess of haem over non-haem Fe. This is due partly to very high and variable 'blanks' caused by solution of haem pigment in the aqueous acid ethanol medium, and partly to a significant decomposition of haem pigment in even the shortest possible heating period. Moreover, in the determination of total Fe in whole blood or liver, the decomposition of haem pigments by the amount of H_2O_2 recommended here only proceeds quantitatively if the specimen has been previously heated to 100°. Preliminary experiments indicate that the non-haem iron method is perhaps exceptionally well suited to the determination of plasma Fe.

When small quantities of Fe are being determined, the care of glassware is very important. Glass easily becomes contaminated with iron which is not, in the author's opinion, satisfactorily removed by the usual $\text{K}_2\text{Cr}_2\text{O}_7$ - H_2SO_4 mixtures. In this laboratory glass is first scrubbed with detergent and then, immediately before use, rinsed with hot HCl or HNO_3 and freed from acid with distilled water only.

RESULTS

The analytical techniques have been applied to seventy-four eggs incubated for periods varying between 4 and 20–21 days. The results are shown in Figs. 1–4, where the wet weight of the embryos is plotted against haem iron in the embryo (Fig. 1), haem iron in the membranes (Fig. 2), haem iron in the whole egg (Fig. 3) and non-haem iron in the embryo (Fig. 4). A double logarithmic scale was chosen for convenience in presenting the results, since the embryo weights vary over the very wide range of 0.15–34 g. This method of plotting, moreover, may allow the detection of certain general trends which are very much less obvious when ordinary arithmetical scales are used.

Fig. 1 shows that the haem iron in the embryo body increases steadily from about 1.2 $\mu\text{g.}$ at embryo weight 0.15 g. to 10 $\mu\text{g.}$ at embryo weight 1 g., 120 $\mu\text{g.}$ at 10 g. and 300 $\mu\text{g.}$ at 20 g. At the point of hatching (embryo weight 30 g. or over) most embryos probably contain between 600 and 750 $\mu\text{g.}$ haem iron. Over the greater part of the incubation period there is a fairly strict proportionality between the two sets of logarithms.

Fig. 2 shows, as might be expected, a very different state of affairs with regard to the membranes. The membrane haem iron increases, but less steadily than that in the embryo body, until it reaches a maximum of 200–300 $\mu\text{g.}$ at embryo weight 16–19 g. After this it begins to decrease

extremely rapidly, until by the time the embryo weighs 30 g., the haem iron in its membranes has dropped to 60 $\mu\text{g.}$, no more than it is at the end of the first week of incubation.

Fig. 1. Embryo haem iron during embryo growth. Note the use of logarithmic scales. ●, points from the analysis of individual embryos; ○, points from the analysis of three to six pooled embryos.

Fig. 3. Total (embryo + membranes) haem iron during embryo growth. Note the use of logarithmic scales.

When these two sets of figures are combined, as in Fig. 3, to show the increase of total haem iron with embryo weight, it can be seen that the proportionality between the two sets of logarithms is broadly maintained, at least until the sharp de-

crease in membrane haem iron becomes apparent towards the end of incubation.

Fig. 4 shows the increase in non-haem iron in the embryo body plotted against embryo weight. It will be observed that there is a broad proportionality between the logarithms of the two quantities

Fig. 2. Membrane haem iron during embryo growth. Note the use of logarithmic scales, and that embryo weight, not membrane weight, is the standard of comparison.

Fig. 4. Non-haem iron in the embryo during growth. Note the use of logarithmic scales. ●, points from the analysis of individual embryos; ○, points from the analysis of three to six pooled embryos.

during the greater part of incubation, but that during the last few days, after the embryo weight exceeds 20 g., there is a sharp increase in the rate of accumulation of non-haem iron. Before this stage,

the embryo contains approximately $7 \mu\text{g./g.}$ non-haem iron, while by the time the weight has reached 25 g. the figure is about $10 \mu\text{g./g.}$, and this increases even more by the time the chick is ready to hatch.

It has already been noted that haem iron in the 'rest' is assumed to be derived from the haemoglobin of the blood circulating in the membranes. Although the 'rest' in these preparations included the amnion, this membrane is so much smaller and less vascular than the others that the haem iron in the 'rest' may be safely regarded as wholly derived from the circulation in the yolk sac and the chorioallantois. The relative importance of the two latter membranes in this respect has been the subject of eight experiments on eggs between the 10th and 16th days of incubation, a period during which the membrane circulations appear to reach their zenith. In these experiments the frozen egg-contents were separated into three parts: the embryo, the yolk with its adherent yolk sac, and the remaining parts—residual white, fluids and chorioallantois. Analyses done on these fractions suggest that at this period of development the circulation of the chorioallantois usually carries nearly as much blood as that of the yolk sac. In six experiments the chorioallantois contained 38–49% of the blood in the membranes, but in the remaining two the values were only 23 and 25%. In these eggs, however, the volume of the allantoic fluid (and therefore presumably the size of the containing membrane) was very much less than in the others.

The relation of embryo wet weight to incubation period was much the same in these experiments as in the work of Byerly (1932), except that in this laboratory the weight at hatching has been lower by perhaps as much as 10%.

DISCUSSION

It is perhaps noteworthy that the average total iron content of these eggs (1.23 mg./egg in 77 eggs; $\text{s.d.} \pm 0.2$) was significantly higher than that of the earlier series (Ramsay, 1950; 1.07 mg./egg in 115 eggs; $\text{s.d.} \pm 0.2$). Inquiry showed that the diet of the flock had been altered in the interval, but unfortunately in several different respects. With the increased iron content of the new series of eggs, the effect of quantity of iron available on quantity of haem synthesized was much less pronounced than before.

It would appear from the results which McFarlane & Milne (1934) quoted that the total iron in the present series of eggs was probably much the same as in those used by the American authors. They separated the embryo from the rest of the egg after snipping the yolk stalk and its attendant vessels as close to the embryo body as possible, and deter-

mined the total iron in the embryo only by wet ashing and thiocyanate colorimetry or titanium titration. Their results agree fairly well with those obtained in the comparable portions of the present work.

Since the circulations of the yolk sac and the chorioallantois are the channels through which the embryo breathes, excretes, and is fed, one might expect that they would increase in magnitude throughout the course of incubation. In point of fact this does not seem to be the case, as may be seen from Fig. 2, or in more striking terms from Fig. 5,

Fig. 5. Membrane haem, as quantity per unit wet weight of embryo ($\mu\text{g./g.}$), during incubation of hen eggs.

which shows a steady decrease in the quantity of membrane haem per unit weight of embryo body between the 9th and 21st days of incubation. Such figures cannot be related to blood volume changes unless the trend of blood haemoglobin concentration figures is also known. Haemoglobin concentrations in chick embryo blood have not yet been undertaken in this laboratory, but a series of analyses was published by Zorn & Dalton (1937). Calculations made with the aid of their figures suggest that the total membrane blood volume may be about 0.3 ml. in 9 days, 0.45–0.5 ml. at 13–17 days, and perhaps less than 0.2 ml. at 20 days. It appears likely that at 9 days, when the embryo is growing at the rate of about 0.15 g. dry weight per day, there is more blood in the membranes than towards the end of incubation, when the absolute rate of growth may be four or five times greater. A somewhat similar state of affairs was encountered by Barcroft and his collaborators in the investigation of foetal physiology in the sheep (Barcroft, 1946). In that species the proportion of the total blood volume which is present in the placenta decreases from 50% between 90 and 100 days to about 15% at 140 days, 1 week before parturition. The absolute volume of

blood in the placenta changes but little during the same period, in spite of the growth of the foetus. Barcroft and his colleagues demonstrated experimentally the truth of the most probable explanation, that the lack of increase in placental blood volume is compensated by a substantial rise in

Fig. 6. Percentage of total embryonic haem iron or blood present in the embryo body at different stages of development. Stage of development expressed as percentage of total incubation or gestation period. (100% = 21 days for hen, 147 days for sheep.) ●—●, points for the hen, calculated from this work; ○, points for the sheep, calculated from results quoted by Barcroft (1946).

arterio-venous pressure difference and a concomitant rise in the rate of circulation. It would be interesting to know whether the chick embryo deals with its related problem in the same way. Fig. 6 illustrates the general similarity between sheep and fowl in the matter of blood distribution between the embryo and its physiological appendages. In both cases about two-thirds of the development time elapses before the embryo body contains half of the total embryonic blood.

Inspection of Fig. 2 poses the question of the fate of the haemoglobin which leaves the membrane circulations during the last few days of incubation. The absence of a sharp upward turn in Fig. 1 at the point corresponding to that at which the membrane haem begins to decrease shows that the rate of increase in the embryo body is insufficient to explain the disappearance of the membrane haem by a process of transference into the embryo body, unless it is assumed that the haemopoietic process is conveniently interrupted for a few days.

Such an occurrence would seem highly improbable, and two further facts point towards a rather different course of events: the late embryo or newly hatched chick has a gall-bladder distended with highly pigmented bile, and during the last few days of incubation, as may be seen from Fig. 4, the rate of accumulation of non-haem iron in the embryo body shows a sharp upward trend. In this respect the non-haem iron behaves differently not only from haem iron, but also from such diverse compounds as choline, creatine, methionine and the nucleic acids (G. S. Boyd, T. M. Sutherland, private communications). It seems highly probable, therefore, that towards the end of incubation haemoglobin degradation is either initiated or accelerated in such a way that the 'slack' created by the disappearance of the membranes is neatly taken up. The iron liberated is retained in the embryo body, where it presumably makes a substantial addition to the stored iron of the young chick.

SUMMARY

1. Total and non-haem iron have been determined in the embryo and the remaining portions of fertile eggs frozen and dissected after incubation for various periods.

2. Haem iron in the embryo body increases in such a way that the logarithm of the amount present is directly proportional to the logarithm of the wet weight. This relationship holds until the last 2 or 3 days of incubation. The quantity present is about 10 $\mu\text{g.}$ at embryo weight 1 g., 300 $\mu\text{g.}$ at 20 g., and 600–750 $\mu\text{g.}$ at 30–35 g.

3. Haem iron in the membranes increases to a maximum of 200–300 $\mu\text{g.}$ at embryo weight 16–19 g., and then decreases sharply. While the embryo increases in weight from 3 to 18 g., the proportion of the membrane haem in the chorioallantois remains about 40%. The proportion of the total haem which is present in all the membranes decreases from 75% at embryo weight 2 g. to 50% at 14 g. and 10% at 30–35 g. A comparison is made between these figures and the state of affairs in the uterus of the pregnant sheep, as described by Barcroft (1946).

4. Evidence is presented that towards the end of incubation a quantity of haem is destroyed which is broadly equivalent to that disappearing from the membranes. The liberated iron accumulates in the embryo, presumably as a store.

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Studies in Vitamin A

15. THE VARIATIONS IN THE SERUM β -CAROTENE AND VITAMIN A LEVELS OF COWS NEAR PARTURITION

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Kuhlmann & Gallup (1944), Braun (1945*a*) and Sutton, Kaeser & Soldner (1945), working in the United States, have reported that at or immediately after parturition the serum carotenoid and vitamin A levels of cows drop sharply, to recover slowly during the next 14–21 days. It was considered important to find out whether such a situation obtained with cows kept under normal English farming conditions, and to this end the work reported here was carried out during 1948.

EXPERIMENTAL

Animals. The cows used in this investigation belonged to the Veterinary Science Division of the Research Department of Boots Pure Drug Co. They formed a small mixed herd used for a long-term experiment on the spread of *Streptococcus agalactiae* infection, but this experiment cannot be considered to affect in any way the vitamin A and carotenoid metabolism of the cows. The general details of the experimental animals are given in Table 1. During the period of the investigation (May–November, 1948) all the cows except no. 7 were out at grass day and night until

17 October, having been turned out on 11 April. Cow no. 7 was kept in after early October because of an illness; after 17 October cows nos. 2, 3 and 7–9 were out at grass only during the day (approx. 5 hr.) and received a daily supplement of third-year seeds hay, up to 20 lb. (9.1 kg.) of kale and concentrates according to milk yield. Cows nos. 4–6 remained out day and night until the investigation ended. On calving the cows received a supplement of 8 lb. (3.6 kg.) per day of a production ration consisting of 1 part bran, 1 part oats, 1 part flaked maize (Kosistos) and 1 part dairy nuts. Throughout the period under investigation there was always plenty of grass available, for, owing to the wet summer, the pasture never dried up.

Determination of serum β -carotene and vitamin A. Blood samples were drawn from the jugular vein into McCartney bottles and immediately posted to Liverpool, where they arrived in good condition, showing little or no haemolysis. The serum was separated from the clot by centrifugation and stored at 0° until it was assayed. Determinations were always carried out as soon as possible after arrival and never more than 48 hr. later. It was assumed that no loss of carotenoids and vitamin A had occurred in transit. The carotenoids and vitamin A were extracted from the serum by the method in general use in this laboratory (Glover, Goodwin & Morton, 1947*a*). Owing to the very high concentration of β -carotene in the extracts, direct determinations of vitamin A were impracticable, because any correction applied

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Table 1. *Details of the cows used during the present investigation*

Cow no.	Date of birth	Breed	No. of days dry before calving	Date of calving
1	Jan. 1944	Ayrshire $\times$	57	30 Apr. 1948
2	Jan. 1944	Ayrshire $\times$	113	30 May 1948
3	Dec. 1944	Ayrshire	1st calf	6 Sept. 1948
4	Mar. 1945	Ayrshire	1st calf	8 Sept. 1948
5	Dec. 1944	Ayrshire	1st calf	21 Sept. 1948
6	Jan. 1945	Shorthorn (Pedigree)	1st calf	15 Nov. 1948
7	Jan. 1945	Ayrshire	1st calf	21 Nov. 1948
8	Feb. 1944	Ayrshire (Pedigree)	1st calf	Feb. 1948
9	Oct. 1944	Ayrshire	1st calf	Feb. 1948