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The influence of ovarian activity or treatment with oestradiol dipropionate on the vitamin B_{12} content of cells and plasma of the blood of the domestic fowl

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At the onset of lay, the hen must provide for the continuous output of nutrients in the eggs. It is well known that the levels of some of the vitamins in the blood increase when the hen begins to lay. Common, Rutledge & Bolton (1946) noted a marked rise in the serum riboflavin content of laying hens, which could also be evoked in the immature pullet by injection of oestradiol dipropionate. In their investigation, the content of serum carotenoids also rose after the oestrogen treatment. Hertz, Dhyse & Tullner (1949) induced similarly a rise in the biotin level of plasma. In view of these observations, we wanted to know whether increased ovarian activity during egg production brought about any changes in the level of vitamin B_{12} in the blood. Accordingly the vitamin B_{12} content of cells and plasma was determined in pullets before and after they had begun to lay and in immature pullets and cockerels receiving oestradiol dipropionate.

The blood of birds differs from that of mammals in having nucleated erythrocytes. Thus, there is the possibility that deoxyribosides (with growth activity for *Lactobacillus leichmannii*) are released from the nucleic acids in the erythrocytes during the preparation of extracts of the blood for microbiological assay of vitamin B_{12}. The blood of the domestic fowl also has the property of combining with large quantities of added vitamin B_{12}, and the vitamin B_{12} naturally present in it is in the form of a protein complex not readily available to assay micro-organisms (Gregory & Holdsworth, 1959). For these reasons, routine methods for measuring vitamin B_{12} in mammalian blood could not be used, and different methods for extracting and measuring the vitamin in the blood of the domestic fowl were therefore investigated. The method finally chosen as being most suitable was then used to study the effect of onset of lay, or of administration of oestrogens to immature birds, on the vitamin B_{12} levels in the blood plasma and cells.

EXPERIMENTAL

Procedure

Expt 1. *Vitamin B_{12} content of whole blood.* Three different methods for extracting the vitamin B_{12} activity from whole blood were investigated. Methods 1, 2 and 3, described on p. 585, were each used to prepare extracts from a sample of pooled blood from 4-week-old pullets. The vitamin B_{12} activity of the extracts, before and after treatment

with alkali (see p. 585), was measured with *Lb. leichmannii* ATCC 4797 by the method of Gregory (1954).

Expt 2. Vitamin B_{12} content of cells and plasma. Blood samples from two groups of five pullets were separated into plasma and cells as described on p. 585. The vitamin B_{12} activity of the cells and plasma of group 1 was extracted by the papain digestion method 3, whereas that of group 2 was extracted by method 3*a*. The vitamin B_{12} activity of these extracts, before and after treatment with alkali, was measured with *Lb. leichmannii* ATCC 4797 (Gregory, 1954).

Expt 3. Effect of oestradiol dipropionate treatment on vitamin B_{12} content of cells and plasma. Two groups of five Light Sussex pullets of about equal body-weight were each placed in a large wire-floored cage at 12 weeks of age and given a commercial diet containing 12μg vitamin B_{12}/kg. The birds in one group were each given an intramuscular injection of 5 mg oestradiol dipropionate in sterile oily solution every 3rd day, so that each received a total of 25 mg over a 2-week period; one pullet died during this period. The other group was not treated. On the 14th day, blood samples were taken from both groups for vitamin B_{12} assay. The untreated pullets were later placed in individual laying cages, and further blood samples were taken when they were 24 weeks of age and had been in lay for about 2 weeks. The vitamin B_{12} contents of eggs collected in the 2nd week were measured.

Two groups of five Light Sussex cockerels were treated in the same way. Blood samples were taken from both groups on the 14th day and also from the birds in the untreated group at 24 weeks of age, when they were mature, as judged by their combs and their beginning to crow.

The vitamin B_{12} contents of the blood cells and plasma of the treated and untreated birds were measured with both *Lb. leichmannii* ATCC 4797 (Gregory, 1954) and *Ochromonas malhamensis* (Ford, 1953) in extracts prepared by method 3*a*. The vitamin B_{12} content of the whole blood was calculated from the values for cells and plasma as:

$$\frac{C \times V}{100} + \frac{(100 - V) \times P}{100},$$

where C and P are the vitamin B_{12} contents of cells and plasma respectively, expressed in mμg/ml, and V is the packed cell volume expressed as a percentage of the volume of whole blood.

The percentage of vitamin B_{12} in the plasma was calculated as:

$$\frac{P \times (100 - V)}{WB},$$

where WB is the vitamin B_{12} content of whole blood expressed in mμg/ml.

The weight of each bird was recorded at intervals during the experimental period, and from the mean body-weights of the different groups at 14 and 24 weeks of age the approximate blood volumes were estimated by the method of Sturkie (1954), on the assumption that treatment with oestrogen had no effect on the blood volume of the bird. An approximate value for the total amount of vitamin B_{12} present in the blood was obtained from the approximate blood volumes and the mean values for the

vitamin B_{12} content of whole blood given in Table 3. The daily food intake/bird was also measured over the 2-week period before blood samples were taken, and from it and the known amount of vitamin B_{12} in the diet (12 μg/kg) the mean daily intake of the vitamin by the birds in each group was estimated.

Preparation of blood samples

Samples were taken from a wing vein into heparinized graduated tubes. Except in Expt 1, in which whole blood was used, the samples were centrifuged for 20 min at 3400 *g* and the volumes of packed cells and plasma were recorded. From these volumes the percentage of packed cells per unit volume of whole blood was calculated. Most of the plasma was removed from the cells by decanting and the remainder with filter-paper. The cells were then washed into a flask with water.

Extraction methods

Method 1: autolysis. The method was based on that of Rosenthal & Brown (1954). Whole blood (5 ml) was laked with 15 ml water, 2 ml toluene were added, and the mixture was incubated at 37° for 18 h. One drop of 1% (w/v) NaCN solution was then added, the pH adjusted to 5·1, and the autolysed blood extract heated in flowing steam for 30 min. After cooling, the volume was made to 50 ml and the extract filtered. The filtrate was diluted to contain about 0·04 mμg vitamin B_{12}/ml and added to the assay tubes in quantities of 0·5, 1·0, 2·0 and 4·0 ml.

Method 2: steaming with cyanide. The procedure was the same as in method 1 except that no toluene was added and the incubation at 37° for 18 h was omitted.

Method 3: papain digestion. This method was the same as method 3*a* described below, but without the preliminary heat treatment.

Method 3a: papain digestion. Plasma (1 ml) was measured into about 40 ml water containing one drop of 0·1% (w/v) NaCN solution and heated in flowing steam for 10 min. After cooling, the pH was adjusted to 4·6 with 0·1 N-HCl and two drops of the NaCN solution were added, then 20 mg papain (British Drug Houses Ltd) in 2 ml water containing a trace of cyanide. The mixture was incubated for 2 h at 55°. Then, after being heated in flowing steam for 10 min, the digest was cooled, made up to a vol. of 50 ml and filtered. The filtrate was further diluted to contain about 0·04 mμg vitamin B_{12}/ml before being added to the assay tubes as in method 1. The cells were treated in the same way, except that it was found necessary to homogenize the extract after the preliminary heating to ensure that the coagulated proteins were finely dispersed before the papain was added.

Alkali treatment of extracts. Vitamin B_{12} is destroyed by heating with alkali (Smith, 1955). To distinguish the true vitamin B_{12} activity of the extracts from that due to deoxyribosides, the extracts were treated with alkali as described previously (Gregory, 1954).

Measurement of vitamin B_{12} in eggs

The contents of the egg were homogenized. A 5 g portion of the homogenate was then treated by extraction method 3*a*, but with a larger quantity of papain (100 mg) activated with two drops of 1·0% (w/v) NaCN solution.

Statistical treatment of results

The significance of the differences between values for (*a*) immature and mature pullets and cockerels and (*b*) oestrogen-treated immature pullets and cockerels was assessed by Student's *t* test at the 1 % level.

RESULTS

Expt 1. *Vitamin B_{12} content of whole blood*

Autolysis or papain digestion of the unheated whole blood released considerable quantities of vitamin B_{12}-like growth activity for *Lb. leichmannii* (see Table 1). A high proportion of this activity was, however, stable to the alkali treatment and was therefore not due to vitamin B_{12}. When the whole blood was heated in the presence of cyanide (by extraction method 2) without any autolysis occurring, no alkali-stable growth

Table 1. *Expt* 1. *Vitamin B_{12} activity (mμg/ml) of chicken whole blood measured with* Lactobacillus leichmannii *after different extraction treatments*

Method of extraction	Vitamin B_{12} activity: Before alkali treatment	After alkali treatment	True vitamin B_{12}
1. Autolysis	7·2 (18)	4·6	2·6
2. Steaming with cyanide	4·2 (33)	0	4·2
3. Papain digested (no preliminary heat treatment)	18·9 (50)	18·9	0?

Figures in parentheses are the percentage recoveries of the cyanocobalamin added before the extraction treatment.

activity was found in the extract. This observation indicated that enzymes present in the blood or in the papain preparation were probably breaking down the nucleic acids in the red cells to form deoxyribosides that have growth activity (stable to alkali) for *Lb. leichmannii*. Although there was no alkali-stable growth activity in the extracts prepared by method 2, the recovery of vitamin B_{12} added to the blood before treatment was poor (see Table 1). In previous investigations at this Institute, it has been found that digestion with papain was necessary to release full vitamin B_{12} activity from chicken serum (Gregory & Holdsworth, 1959). Therefore, in Expt 2 the blood was separated into plasma and cells, and a modification of the papain digestion method was tried.

Expt 2. *Vitamin B_{12} content of cells and plasma*

The results are shown in Table 2. As was expected, when the extracts of cells and plasma were prepared by method 3 (i.e. by papain digestion of the unheated samples), all the alkali-stable growth activity was associated with the cells (see Table 2, group 1). But, although all the activity measured in the plasma was due to vitamin B_{12}, recovery of cyanocobalamin added to one of the samples (from pullet 18) was poor, indicating that not all the binding activity had been destroyed and possibly that not all the natural vitamin B_{12} had been released. However, little or no alkali-stable growth activity was present when the extracts of the cells were prepared by method 3*a*, that is, by digestion

with papain after preliminary heat treatment (see Table 2, group 2). Recovery of vitamin B_{12} added to a portion of one of the samples (from pullet *Y*) was satisfactory. Similarly, 118 and 106% of the added cyanocobalamin were recovered from two of the plasma samples. It is clear from these results that release of deoxyribosides from the red cells does not occur if the blood enzymes are inactivated before addition of the papain.

Table 2. *Expt 2. Vitamin B_{12} content (mμg/ml) of pullet blood cells and plasma, measured with* Lactobacillus leichmannii *after papain digestion of the unheated samples (group 1) or of the heat-treated samples (group 2)*

		Cells			Plasma		
Pullet no.	Treatment before papain digestion	Untreated extract	Alkali-treated extract	Recovery*	Untreated extract	Alkali-treated extract	Recovery*
Group 1							
13	None (method 3)	4·3	3·2	—	4·3	0	—
14		2·9	2·0	—	5·0	0	—
15		4·1	3·4	—	3·8	0	—
16		5·2	4·4	30	4·6	0	—
18		15·8	—	0	5·8	0	40
Group 2							
U	Heated in flowing steam for 10 min (method 3*a*)	1·6	0	—	6·25	0	—
V		1·7	0	—	3·60	—	—
W		3·6	0	—	3·25	—	118
X		1·9	0·4	—	4·25	0	—
Y		1·9	0·3	84	4·85	0	106

* Percentage recovery of vitamin B_{12} added before any extraction treatment.

Expt 3. Effect of oestradiol dipropionate treatment on the vitamin B_{12} content of cells and plasma

Method 3*a* was used in this experiment to extract the vitamin B_{12} activity from blood cells and plasma of untreated pullets and cockerels and those treated with oestradiol dipropionate, since the results of Expt 2 had shown that it was the most satisfactory. The results are presented in detail in Table 3, which also gives values for the vitamin B_{12} content of whole blood and for the percentage of vitamin B_{12} in the plasma, calculated as described on p. 584. Only results obtained with *Lb. leichmannii* as the test organism are given. The low potency of some of the samples made assay with *O. malhamensis* difficult, and often results had to be calculated from values obtained at only one level of added test extract. However, the results obtained with this organism were in broad agreement with those obtained with *Lb. leichmannii*.

The calculated vitamin B_{12} content of whole blood was markedly higher in laying than in immature pullets. The difference was accounted for by a significantly higher ($P < 0{\cdot}01$) level in the plasma and a smaller but still significant ($P < 0{\cdot}01$) increase in the level in the cells.

Administration of oestradiol dipropionate to the immature pullets resulted in no significant change in the vitamin B_{12} content of whole blood. However, there was a marked decrease in percentage cell volume, and as a result the distribution of vitamin B_{12} between the blood cells and plasma became similar to that in the laying pullet.

The vitamin B_{12} levels in the blood of mature and immature cockerels were not significantly different. Treatment of immature cockerels with oestradiol dipropionate caused a significant increase in concentration of vitamin B_{12} in the plasma, whereas that in the packed cells remained unchanged. At the same time a marked decrease in percentage cell volume occurred, and consequently the distribution of vitamin B_{12}

Table 3. *Expt* 3. *Vitamin B_{12} measured with* Lactobacillus leichmannii *in the cells and plasma of the blood of immature and mature pullets and cockerels and of immature pullets and cockerels treated with oestradiol dipropionate*

Age (weeks)	Stage of maturity	Dose of oestradiol dipropionate	No. of birds	Cell volume (%)	Mean vitamin B_{12} content (mμg/ml) Plasma	Cells	Whole blood†	% of vitamin B_{12} in plasma†
				Pullets				
14	Immature	None	5	33	1·5	1·1	1·3	77
14	Immature	25 mg	4	23	2·0	1·2	1·8	86
24	Laying	None	5	27	4·0**	1·8**	3·4	86
				Cockerels				
14	Immature	None	5	35	1·0	1·3	1·1	59
14	Immature	25 mg	4	22	2·1**	1·2	1·8	91
24	Mature	None	5	43	0·9	1·6	1·2	43

† For calculation see p. 584.
** Significantly different ($P < 0·01$) from that in untreated immature birds.

Table 4. *Mean values for body-weight, estimated volume and total vitamin B_{12} content of blood, and daily food and vitamin B_{12} intake of immature and mature pullets and cockerels and immature pullets and cockerels treated with oestradiol dipropionate*

Age (weeks)	Stage of maturity	Dose of oestradiol dipropionate	No. of birds	Body-weight (g)	Blood: Approx.* volume (ml)	Blood: Total* vitamin B_{12} content (mμg)	Food intake per bird† (g)	Vitamin B_{12} intake (mμg)
				Pullets				
14	Immature	None	5	1635	134	174	110	1320
14	Immature	25 mg	4	1713	138	248	145	1740
24	Laying	None	5	2575	180	612	200	2400
				Cockerels				
14	Immature	None	5	1785	171	188	115	1380
14	Immature	25 mg	4	1815	174	313	146	1752
24	Mature	None	5	2860	263	316	168	2016

* For calculation see p. 584.
† Measured over the 2-week period before blood samples were taken.

between the cells and plasma in these oestrogen-treated cockerels was almost the same as that in the oestrogen-treated immature pullets and similar to that in laying pullets.

Mean values for body-weight, calculated volume and total vitamin B_{12} content of the blood and for daily food and vitamin B_{12} intakes of the different groups of birds are

given in Table 4. It can be seen that the quantity of vitamin B_{12} in the diet was more than enough to supply the increased amounts of the vitamin present in the blood of oestrogen-treated immature birds or of laying pullets.

Vitamin B_{12} content of eggs

The eggs contained from 320 to 710 mμg vitamin B_{12}/egg, the mean value being 460 mμg.

DISCUSSION

The striking increase observed in the vitamin B_{12} content of pullet blood at the onset of lay was similar to the increases in riboflavin, carotenoids and biotin observed by others (Common *et al.* 1946; Hertz *et al.* 1949). It would seem that this increase is due to a specific adaptation of the laying bird to meet the demands of egg production rather than that it represents just a rise with increased maturity and growth. That it was so is shown by our finding that there was no corresponding increase in the vitamin B_{12} content of the plasma, or indeed of the whole blood, of mature cockerels of similar age. Further, it was possible to evoke a rise in the level of vitamin B_{12} in the plasma of immature cockerels and pullets by administration of oestradiol dipropionate. However, this rise was not so marked as that which occurred in the pullets at the onset of lay. Probably a greater increase would have resulted for a mixture of hormones more closely resembling those active in the blood.

A further effect of the oestradiol dipropionate treatment was that marked changes occurred in the percentage of packed cells per unit volume of blood. The decrease observed in oestrogen-treated pullets and cockerels may have been due to a decrease in the number of red cells. Domm & Taber (1946) found that both capons and young oestrogen-treated pullets had fewer erythrocytes than their controls. On the other hand, the decrease in cell volume may merely have reflected an increase in blood volume; Common *et al.* (1946) were of the opinion that oestrogen treatment induced a marked increase in blood volume. The greater proportion of vitamin B_{12} in the plasma of laying pullets and of oestrogen-treated immature pullets and cockerels was partly due to the decrease in cell volume observed in these groups. The large cell volume we found in the blood of mature cockerels agrees with values given in the literature (see Sturkie, 1954) and accounts for the higher proportion of vitamin B_{12} in the cells of these birds.

The only other reported study of the distribution of vitamin B_{12} between cells and plasma of chicken blood is that of Rosenthal & Brown (1954). From a haematocrit value of 29% and values of 6·53 mμg vitamin B_{12}/ml whole blood and 1·06 mμg/ml plasma, they calculated that only 12% of the total vitamin activity was in the plasma. This is contrary to our findings reported here. The explanation probably lies in the fact that Rosenthal & Brown autolysed their samples as a part of the extraction treatment. Our comparison of different methods for extracting vitamin B_{12} activity from blood showed that, although chicken blood requires some enzyme treatment to release the vitamin from its bound form, any procedure that allows autolysis to occur is unsatisfactory, since it results in deoxyribosides, with growth activity for *Lb. leichmannii*, being present in the extract. We found that preliminary heat treatment of the blood

cells or plasma, to inactivate the natural enzymes and denature the protein, before treatment with papain gave maximum release of the vitamin without the appearance of accompanying non-specific growth factors.

The immediate source of vitamin B_{12} for the egg is not known, though it would seem that the pullet rapidly mobilizes reserves in the liver and at the same time absorbs considerably larger quantities from its food. The food intake of the oestrogen-treated pullets and cockerels was greater than that of their controls. We calculated that the daily intake of vitamin B_{12} from food was ample for supplying the total increase in vitamin B_{12} content of the blood. Similarly, the daily food intake of the laying pullet would have provided sufficient vitamin B_{12} to maintain the increased total of 612 mμg vitamin B_{12} circulating in the blood. Since the eggs produced by these pullets contained from 320 to 710 mμg vitamin B_{12}/egg, there must be a complete turnover of the vitamin B_{12} content of the blood during deposition of the vitamin in the egg.

SUMMARY

1. Different methods for extracting and measuring vitamin B_{12} in chicken blood have been investigated. Maximum release of the vitamin, without accompanying release of non-specific growth factors for the test organism (*Lb. leichmannii*), was obtained by heating the blood before digesting it with papain. This method was therefore used to study the vitamin B_{12} content of the blood of immature and mature pullets and cockerels and of immature pullets and cockerels treated with oestradiol dipropionate.

2. Groups of five immature pullets and cockerels were injected with oestradiol dipropionate (25 mg over a 2-week period), and the vitamin B_{12} content of the blood was measured and compared with that of the blood of groups of five untreated immature and mature birds.

3. At the onset of lay there was a significant increase in the vitamin B_{12} content of pullet blood. To a lesser extent this increase was evoked in the blood of immature pullets and cockerels by treatment with oestradiol dipropionate. There was no corresponding change in the vitamin B_{12} content of the blood of cockerels as they reached maturity.

4. The distribution of vitamin B_{12} between blood cells and plasma was also measured. In the mature pullet, 86% of the total vitamin B_{12} activity (3·4 mμg/ml) was in the plasma, whereas in mature cockerels only 43% of the total vitamin B_{12} (1·2 mμg/ml) was present in it.

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