Vitamin B_{12} in the developing chick embryo

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It is well established that bacteria are the chief source of vitamin B_{12}, and that higher plants and animals are generally unable to synthesize the vitamin. The work of Woolley (1955) on mammary cancer tissue of the mouse suggests, however, that certain animal tissues may have this ability. It seemed therefore that, by analogy, conditions might be favourable for the synthesis of vitamin B_{12} in the developing chick embryo.

Although Mikata (1953), using *Euglena gracilis*, has shown that the amount of vitamin B_{12} in eggs did not change in the course of 20 days' incubation, studies with the same organism at Cracow showed an increase during development of the embryo. These preliminary observations were brought to the notice of one of us (S.K.K.) during his visit to Poland, and it was arranged to investigate the problem more fully in collaborative work at the two laboratories. The joint findings are presented here. While this paper was being prepared, Fischer, Benson & Swendseid (1958) reported an increase during incubation in the vitamin B_{12} content of eggs as measured by *Lactobacillus leichmannii*. Their findings and those of Mikata are discussed later.

EXPERIMENTAL

At Cracow

Source of material

The eggs used were from Sussex hens mated to Polish Greenleg cocks and fed on a mixed diet containing animal protein. They were incubated in a forced-draught incubator at 39° and at a suitable relative humidity. Samples of eggs were taken after 0, 7, 11, 14, 18 and 20 days of incubation.

Preparation of samples

General. Each egg was analysed separately. In one experiment the whites were separated from the yolks of fresh eggs and in the others the whole egg contents were used.

Fresh eggs. The yolks, whites or whole egg contents were homogenized individually in a Waring Blendor and diluted to 1000 ml. with 0·001 M-KCN in 0·9% (w/v) NaCl solution. The homogenate was brought to pH 7, and a 10 ml. sample was autoclaved at 110° for 1 h. The supernatant liquid was decanted from the coagulated proteins and

diluted to contain from 1 to 6 $\mu\mu$g of vitamin B_{12}/ml. for assay with *E. gracilis* and from 100 to 800 $\mu\mu$g/ml. for assay with *Ochromonas malhamensis*.

Incubated eggs. These were frozen at $-5°$; the shells were removed and the contents homogenized and diluted to 1000 ml. as before, and homogenized again. The freezing of the embryonated eggs was essential for complete comminution of all tissues. Further treatment of the samples was as described for the fresh eggs.

Recovery tests

Two 10 ml. samples of the homogenate of the contents of a fresh egg were taken, and a measured quantity of cyanocobalamin (0·01 μg) was added to one of them. Both samples were then treated as described above and the vitamin B_{12} was measured microbiologically.

Microbiological methods

Vitamin B_{12} was measured with two different test organisms. The method of assay with *O. malhamensis* was as described by Ford (1953) and with *E. gracilis* var. *bacillaris* as described by Ross (1952), but modified by Ostrowski, Skarżyński & Żak (1954) in that growth was measured by extracting the chlorophyll with methanol and measuring the green colour at 665 mμ.

Chromatography

Preparation of extracts. Samples consisting of the contents of ten whole eggs were taken at 0 and 20 days' incubation. Each bulked sample was homogenized with an equal volume of 0·001 M-KCN in 70% aqueous acetone. The homogenates were autoclaved at 110° for 1 h. After centrifuging, the supernatant liquid was concentrated under reduced pressure. The precipitate formed was removed by centrifuging and discarded. The concentrate was adsorbed on to an alumina column (Lens, Wijmenga, Wolff, Karlin, Winkler & De Haan, 1952). The eluate obtained with 5 ml. distilled water was concentrated under reduced pressure to a few drops, which were then placed on filter-paper for chromatography. A sample of crystalline cyanocobalamin was included on the chromatogram.

Analysis. Chromatography was done by the ascending technique on Whatman no. 1 paper. The solution used as the mobile phase was either water-saturated butanol with the paper impregnated with 0·66 M-KH_2PO_4 (Woodruff & Foster, 1950) or 0·1 M sucrose with the addition of 40% isopropyl alcohol (Ostrowski, 1955). The developed and dried chromatograms were cut into transverse 1 cm strips. Each strip was autoclaved at 110° for 10 min in 3 ml. distilled water. The vitamin B_{12} activity of the extracts was then measured microbiologically both with *E. gracilis* and *O. malhamensis*.

At Shinfield

Source of material

The eggs were all produced by one flock of Light Sussex hens mated to Light Sussex cocks and fed on a commercial-type breeder's mash. The vitamin B_{12} content of this mash, as measured by *O. malhamensis* (see below), was 12 μg/kg. The eggs were

incubated at 36·7–37·8° and a relative humidity of 52. Samples of eggs were taken after 11, 14, 18 and 20 days' incubation.

Preparation of samples

General. At each stage of incubation the contents of the eggs (from seven to twelve, see Table 1) were bulked. In one experiment, yolks were removed, bulked and assayed separately from the bulked remainders. In another experiment a sample of bulked whole egg contents was taken for assay.

Yolks. About an equal volume of distilled water was added to the bulked yolks, which were then broken up in a Waring Blendor, and the total weight of homogenate was recorded. A portion (10 g) was weighed out, two drops of 1% (w/v) NaCN solution and 20 ml. water were added, and the pH was adjusted with hydrochloric acid to 4·6. The samples were then autoclaved at 115° for 10 min. The resulting coagulated mass was broken up with a glass rod, made to a volume of 100 ml. with distilled water and homogenized for a few seconds in a Waring Blendor. The solids were then removed by centrifugation. The cloudy supernatant liquid was filtered through a Whatman no. 42 filter-paper and further dilutions were made so that for assay with *Lb. leichmannii* the concentration was about 40 $\mu\mu$g vitamin B_{12}/ml. and for assay with *O. malhamensis* about 200 $\mu\mu$g.

Embryos. After the yolks had been removed, the remainder of the contents of the eggs, comprising the embryos, albumin and embryonic membranes with associated fluid, was bulked and treated in the same manner as the yolks except that the supernatant liquids obtained after centrifuging were not filtered.

Whole egg contents. The whole contents were bulked and homogenized with water, and the total weight of homogenate was recorded. The homogenates were then prepared for assay in the same way as the yolks.

Microbiological methods

Vitamin B_{12} was measured with two different test organisms. The methods of assay were as described by Ford (1953) for *O. malhamensis* and by Gregory (1954) for *Lb. leichmannii* ATCC 4797.

RESULTS

At Cracow

Table 1 shows the mean values and ranges for vitamin B_{12} in whole eggs at 0, 7, 11, 14, 18 and 20 days' incubation. Eggs from the same hen did not vary by more than ± 20% but it can be seen from Table 1 that the range of values for eggs from different hens was much greater. We also noted that, when the vitamin B_{12} content of a fresh egg was high, that of the incubated eggs from the same hen was also high.

Separate analyses of the yolks and whites of fifty fresh eggs showed that the mean vitamin B_{12} content of the yolk was 0·63 μg with a range of 0·38–1·00 μg, and of the white 0·036 μg with a range of 0·015–0·067 μg, as measured with *E. gracilis*.

As measured with *E. gracilis*, the vitamin B_{12} activity of the eggs increased pro-

gressively during incubation. However, with *O. malhamensis* there was no significant increase in the mean vitamin B_{12} activity from 0 to 20 days of incubation.

Table 2 shows the recoveries of vitamin B_{12} added to fresh eggs as measured with

Table 1. *Vitamin B_{12} content (μg/egg) of developing eggs measured at Cracow with* Euglena gracilis *and* Ochromonas malhamensis *and at Shinfield with* O. malhamensis *and* Lactobacillus leichmannii

Test organism	Test material	Day of incubation 0	7	11	14	18	20
		Cracow results*					
E. gracilis	Whole egg	0·88 0·39–1·36 (31)	0·92 0·35–1·87 (12)	1·56 1·08–2·48 (31)	1·65 1·10–2·09 (12)	2·83 1·45–4·22 (8)	3·62 2·11–5·45 (49)
O. malhamensis	Whole egg	0·43 0·13–1·06 (25)	—	—	—	—	0·65 0·48–1·17 (28)
		Shinfield results†					
O. malhamensis	Yolk	—	—	0·37	0·38	0·21	0·09
	Remainder	—	—	0·16	0·18	0·43	0·55
	Total	—	—	0·53 (12)	0·56 (12)	0·64 (12)	0·64 (12)
	Whole egg	0·90 (12)	—	0·57 (12)	0·70 (9)	1·04 (7)	0·65 (7)
Lb. leichmannii	Yolk	—	—	0·35	0·39	0·22	0·09
	Remainder	—	—	0·15	0·17	0·50	0·69
	Total	—	—	0·50 (12)	0·56 (12)	0·72 (12)	0·78 (12)
	Whole egg	1·07 (12)	—	0·85 (12)	0·77 (9)	1·31 (7)	0·96 (7)

The number of individual eggs or the number in the bulked sample is shown in parentheses.

* Mean values and ranges are given for individual eggs.

† These results are the mean of three assays of bulked egg homogenates which were assayed simultaneously with *O. malhamensis* and *Lb. leichmannii*.

Table 2. *Recovery at Cracow in the* Euglena gracilis *test of cyanocobalamin added to fresh egg homogenates*

(10 mμg cyanocobalamin were added to 10 ml. homogenate)

Egg no.	Vitamin B_{12}: Measured before addition (mμg)	Measured after addition (mμg)	Recovery (%)
1	9·2	18·5	93
2	12·7	21·0	83
3	9·8	19·3	95
4	13·7	23·0	93
5	8·9	19·8	109
6	7·8	17·8	100
7	7·0	17·0	100
8	4·5	13·3	88
9	5·5	15·8	103
10	4·5	13·9	94

E. gracilis. The whole of the added vitamin was recovered, within the error of the method, and therefore the results do not indicate the presence in fresh eggs of a factor that might inhibit the growth of *E. gracilis*.

The results of the chromatographic analysis showed that with both solvents and both assay organisms only one zone of activity corresponding to cyanocobalamin was obtained.

At Shinfield

Table 1 shows the results obtained in two experiments at Shinfield when the egg extracts were assayed simultaneously with *O. malhamensis* and *Lb. leichmannii*. In one experiment the yolks were tested separately from the remainder. After the 14th day of incubation, the level of vitamin B_{12} in the yolk decreased and a corresponding increase occurred in the remainder of the egg, which included the embryo. This increase was slightly greater when measured with *Lb. leichmannii* than with *O. malhamensis*. Thus with *Lb. leichmannii* the total vitamin B_{12}/egg, obtained by adding the values for the separate parts, was higher at 18 and 20 days, whereas when the test organism was *O. malhamensis* the sum of the values for the separate parts did not change throughout incubation.

In the second experiment assays after different incubation periods were done on whole egg contents to avoid errors in the measurement of total vitamin B_{12} that may result from assaying the yolks and embryos separately. The results with both test organisms showed no increase in vitamin B_{12} during the 20 days of incubation. The total vitamin B_{12} content of fresh eggs measured at Shinfield fell within the range found at Cracow.

DISCUSSION

The results reported in this paper have been obtained by use of three different test organisms, *O. malhamensis*, *Lb. leichmannii* and *E. gracilis*. Of the three, *O. malhamensis* is the most specific for vitamin B_{12} and, unlike *Lb. leichmannii* and *E. gracilis*, does not respond to other vitamin B_{12}-like factors or deoxyribosides (Ford, 1953). With this test organism, both laboratories agree that there was no increase in the vitamin B_{12} activity of the whole egg during development of the embryo.

Essentially the same results were obtained at Shinfield with *Lb. leichmannii* as with *O. malhamensis*. However, Fischer *et al.* (1958), using *Lb. leichmannii* as the test organism, found that the mean vitamin B_{12} content of incubated eggs increased from 0·12 μg/egg at 0 days to a maximum of 0·40 μg at 14 days and then decreased to 0·21 μg at 20 days' incubation. Their mean values are lower than ours quoted in Table 1. As far as we can gather from their paper, Fischer *et al.* did not use cyanide when preparing the samples for assay. Denton & Kellogg (1953) have shown that the value for the vitamin B_{12} content is several times higher when cyanide is used in the preparation of extracts of fresh eggs than in its absence. If, as seems likely, the low values found by Fischer *et al.* were due to incomplete extraction of the vitamin from their egg samples, then their conclusions about changes in the vitamin B_{12} content of eggs during incubation are open to doubt.

When *E. gracilis* was used at Cracow, it gave values for whole eggs significantly

higher on the 18th and 20th days of incubation than those obtained with *O. malhamensis*. Mikata (1953) also used *E. gracilis* to measure vitamin B_{12} in the developing hen's egg, but did not observe any increase in total vitamin B_{12} activity during 20 days' incubation. His values for the vitamin in fresh eggs (0·09–0·14 μg) were considerably lower than ours, but, as with the results of Fischer *et al.*, this difference could perhaps be explained by the absence of cyanide and by other differences in the extraction procedure.

Added cyanocobalamin was recovered quantitatively from fresh eggs in the *E. gracilis* test. Moreover, the value for fresh eggs obtained with *E. gracilis* was essentially the same as that obtained with *O. malhamensis*, which suggests that the higher results with *E. gracilis* for incubated eggs were due to a substance formed during the development of the embryo and having growth activity for *E. gracilis* but not for *O. malhamensis*. This growth factor is not one of the vitamin B_{12} analogues, since chromatography showed only cyanocobalamin to be present in extracts of fresh or incubated eggs. The reason for the absence from these chromatograms of a substance, other than cyanocobalamin, with growth-promoting activity for *E. gracilis* may be that the method of preparing the extracts for chromatography had not extracted the *E. gracilis* growth factor. Another possibility is that the factor stimulates the growth of *E. gracilis* only in the presence of vitamin B_{12} and not in its absence. A third possibility is that the factor is destroyed during the extraction and chromatography. In this respect it might resemble the *Crithidia fasciculata* factor, which is labile to acid, loses its activity on paper chromatography and is not one of the vitamin B_{12} analogues, although it replaces vitamin B_{12} for *E. gracilis* and *Escherichia coli* but is inactive for *O. malhamensis* (Seaman & Sanders, 1957).

It would seem that *E. gracilis* measures, in addition to vitamin B_{12}, a substance formed during the development of the embryo. In both laboratories the results with *O. malhamensis*, which measures vitamin B_{12} specifically, confirm that there is no increase in the vitamin B_{12} content of hen's eggs during incubation.

SUMMARY

1. Three assay organisms, *Ochromonas malhamensis*, *Euglena gracilis* and *Lactobacillus leichmannii*, have been used in two laboratories to measure the vitamin B_{12} activity of eggs at different stages of development.

2. The results with *O. malhamensis* in both laboratories, and with *Lb. leichmannii* in one, showed no increase in the vitamin B_{12} content of the egg during incubation.

3. The vitamin B_{12} activity for *E. gracilis* increased progressively during incubation of the eggs. Chromatographic analysis and recovery tests suggested that *E. gracilis* was responding to some stimulatory factor, other than any of the vitamin B_{12} analogues, formed during the development of the embryo.

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Growth response to dietary penicillin of germ-free chicks and of chicks with a defined intestinal flora

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In certain circumstances, antibiotics added to the diet increase the rate of growth of chicks (for review see Jukes, 1955). Coates, Dickinson, Harrison, Kon, Porter, Cummins & Cuthbertson (1952) and Coates, Davies, Harrison, Kon & Porter (1955) have suggested that this action of penicillin added to a diet complete in all known essential nutrients may be due to the suppression of an unidentified 'infection' that depresses growth. This view was recently confirmed in a series of experiments with germ-free and conventional chicks (Forbes & Park, 1958). Lev, Briggs & Coates (1956, 1957) observed that spores of *Clostridium welchii* type A were present in the caecums of chicks from the 'infected' premises 1 day after feeding, but not in those from the clean environment. A growth stimulation by penicillin occurred only in the 'infected' premises where the growth rate of chicks was depressed compared with that of the chicks in the clean quarters. Penicillin in the diet either eliminated the clostridia from the intestines of the chicks or reduced the lecithinase production of these organisms. Thus the presence of *Cl. welchii* type A in the caecums of chicks was associated with growth depression, and elimination of the organisms or reduction in their toxigenicity accompanied the reversal by penicillin of the growth depression. Because of the complex nature of the intestinal flora it was not possible by the usual bacteriological techniques to determine the role of clostridia in the growth of chicks. Germ-free chicks, or chicks in which a defined flora had been implanted, seemed to permit a direct approach to the problem.

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