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Vitamin B_{12}-like Compounds

1. Vitamin B_{12} Activity for Chicks and for different Micro-organisms of Gut Contents and Faeces*

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In studying the vitamin B_{12} metabolism of the ruminant we were interested in comparing microbiological and chick measurements of vitamin B_{12} in rumen contents and faeces, and this paper deals with the results of such comparisons.

It has been generally accepted that certain strains of *Lactobacillus leichmannii*, *Bacterium coli* and *Euglena gracilis* can be used for the measurement of vitamin B_{12}. For some time we have used *Lb. leichmannii* and *Bact. coli* for the routine assay of the vitamin in natural materials and have found broad similarity between results obtained with these two micro-organisms. The speed and simplicity of the *Bact. coli* plate assay made it useful for higher-potency materials, whereas the assay of less potent materials was more conveniently carried out in a tube assay with *Lb. leichmannii*. We have also used *Euglena gracilis*, believed to be more specific for vitamin B_{12} than other assay organisms.

There is increasing evidence that the chick assay of vitamin B_{12} in natural materials is not specific for the vitamin, because it measures the whole 'animal protein factor' complex. The results, therefore, are not strictly comparable with those obtained by microbiological methods and tend on the whole to be markedly higher (Coates, Harrison & Kon, 1951).

The application of both chick and microbiological methods to the measurement

* Read in part before the Biochemical Society (Coates, Ford, Harrison, Kon, Porter, Cuthbertson & Pegler, 1951).

of the vitamin B_{12} in gut contents and faeces showed that the microbiological methods disagreed widely among themselves and that, contrary to expectation, the chick assay gave a much lower result than any of the microbiological methods, suggesting the probable presence of substances relatively much more active for micro-organisms than for higher animals.

EXPERIMENTAL

Chick assays

The procedure followed was that described by Coates, Harrison & Kon (1951).

Microbiological assays

Preparation of extracts

Samples of gut contents or faeces (10 g) were mixed with 50 ml. of 1% sodium acetate buffer at pH 4·8 and 0·5 ml. of 1% sodium cyanide was added. The whole was heated in flowing steam for 30 min, cooled, and clarified by centrifugation. The supernatant fluid was of a potency suitable for assay with *Bact. coli*; further dilution (1 : 500) provided extracts for *Lb. leichmannii* assay.

Certain extracts were prepared without the addition of sodium cyanide.

Technique with Lb. leichmannii

For assays with this organism we used essentially the technique and medium of Skeggs, Nepple, Valentik, Huff & Wright (1950), but with the following modifications. The organism was carried in stab culture by monthly transfer in the basal medium, with 2% agar added and supplemented with 0·05 mμg vitamin B_{12}/ml. Inocula were prepared by growing the organism for 18 h at 37° in the same basal medium as is used for the assay, but again supplemented with 0·05 mμg vitamin B_{12}/ml. The culture was then diluted 1 : 100 with physiological saline, and one drop was added to each assay tube. The basal medium was modified only in that nucleotides were omitted and the pH was adjusted to 5·5. It was made up at ten times single strength and stored in polythene bottles at −20° for periods of up to 2 months. The response to vitamin B_{12} was unchanged by this storage, and assays of gut contents and faeces gave similar results on using either freshly made or stored media.

Assays were set up in 19 × 150 mm optically matched tubes. Baskets of the filled tubes were covered with cotton towels which in their turn were covered by aluminium trays, and steamed for 30 min. After cooling, the tubes were inoculated and incubated for 72 h at 35° in an enclosed water-bath fitted with a pump to circulate the water continuously. Responses were measured turbidimetrically.

Standards. Crystalline cyanocobalamin (Glaxo Laboratories Ltd.) was used as a standard over a dose range from 0·01 to 0·2 mμg/tube. Stock solutions containing 10 μg vitamin B_{12}/ml. were made up in 25% ethanol and stored at 2°. The standards were prepared in the following way. To 1 ml. of 10 μg/ml. stock solution was added one drop of a 1% solution of sodium cyanide. Dilution to a concentration of 0·05 mμg/ml. was then carried out in volumetric flasks of brown glass.

Technique with Bact. coli

With this organism we have used the method described by Bessell, Harrison & Lees (1950), Cuthbertson, Pegler & Lloyd (1951) and Harrison, Lees & Wood (1951). The vitamin B_{12} standards and test extracts normally used were intermediary dilutions of those prepared for *Lb. leichmannii* assay.

Technique with Euglena gracilis

Results with this organism were obtained by the technique of Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin & Jukes (1949).

RESULTS

Microbiological assays

Different extraction procedures

Effect of cyanide. The results of assays of rumen contents and faeces presented in Table 1 show that *Lb. leichmannii* gave a markedly lower result than *Bact. coli* and also that the presence of sodium cyanide during the extraction caused a marked increase in the vitamin B_{12} activity measured with both organisms, although the discrepancy between the results with the two organisms remained.

Table 1. *Vitamin B_{12} activities of extracts of calf rumen contents and faeces, prepared with and without cyanide*

	Vitamin B_{12} activity* (μg/g fresh material)			
	Assayed with *Bact. coli*		Assayed with *Lb. leichmannii*	
Substance	Extracted with cyanide	Extracted without cyanide	Extracted with cyanide	Extracted without cyanide
Calf rumen contents (dried) *a*	3·80	2·50	1·10	0·56
b	4·50	2·40	1·75	0·60
Calf faeces (fresh)	1·90	0·40	0·19	0·07

* Expressed as cyanocobalamin.

Table 2 shows that the effect of cyanide is probably to facilitate extraction. A sample of calf faeces was extracted by steaming with 1 % sodium acetate, and the vitamin B_{12} activity of the extract was measured; sodium cyanide did not increase the potency of this extract, but the residue in the presence of sodium cyanide gave a further extract showing vitamin B_{12} activity. The sum of the activities released by these procedures was in agreement with that found in a sample extracted only once, but in the presence of sodium cyanide.

As the highest potencies were obtained by extracting in the presence of cyanide, its addition was made before extraction for all subsequent assays. The results on a number of samples of gut contents and faeces are given in Table 3.

Ultrafiltration. The activity, for both *Lb. leichmannii* and *Bact. coli*, of extracts prepared in the presence of cyanide passed completely through an ultrafilter. However, ultrafiltration of freshly voided faeces diluted with physiological saline showed

that only about 5% of the vitamin B_{12} activity of a sample of calf faeces and only 0·1% of that of a sample of chick caecal contents passed through a Cellophane membrane. This indicates that the activities were largely 'bound', presumably within the bacterial cells.

Table 2. *Effect of cyanide on extraction of vitamin B_{12} activity from a sample of calf faeces*

Preparation of extract	Vitamin B_{12} activity* (μg/g fresh faeces) Assayed with *Bact. coli*	Assayed with *Lb. leichmannii*
Steamed 30 min with 1% sodium acetate, pH 5·0, and centrifuged	1·70	0·32
Cyanide added to extract	1·70	0·31
Residue from extract, re-extracted with addition of cyanide	0·85	0·14
Steamed 30 min with 1% sodium acetate, pH 5·0, and cyanide, and centrifuged	2·50	0·55

* Expressed as cyanocobalamin.

Table 3. *Vitamin B_{12} activity of gut contents and faeces for* Bacterium coli *and* Lactobacillus leichmannii

Substance	Vitamin B_{12} activity* (μg/g) Assayed with *Bact. coli*	Assayed with *Lb. leichmannii*
Calf faeces:		
Fresh *a*	1·9	0·19
b	3·8	1·5
Dried	10·6	1·9
Calf rumen contents:		
Fresh	0·9	0·26
Dried	3·8	1·1
Chick droppings (fresh)	1·0	0·27
Chick caecal contents (fresh)	7·0	1·65
Rat faeces	11	2·1

* Expressed as cyanocobalamin.

Tests for specificity of microbiological measurements

Effect of autoclaving at pH 11·6 on the measured vitamin B_{12} activity. The activity of extracts of gut contents and faeces for both assay organisms was almost wholly destroyed by autoclaving for 15 min at 121°, at pH 11·6. Vitamin B_{12} added to extracts treated in this way (after readjustment of pH to 6·8) was quantitatively measured by either organism.

Recovery of added vitamin B_{12}. Vitamin B_{12} was quantitatively recovered when added before or after extraction of calf rumen contents or faeces.

There was no significant evidence of non-parallelism in growth responses or of the presence of interfering substances. Both the techniques with *Bact. coli* and *Lb. leichmannii* therefore appeared capable of measuring vitamin B_{12} in this type of material.

Assays of vitamin B_{12} activity of Bact. coli *cells*

An explanation of the higher results obtained in the assay of calf faeces with *Bact. coli* as compared with those by the *Lb. leichmannii* method might be that the faeces contained a precursor or metabolite of vitamin B_{12} that the former organism could transform into the vitamin. To test this possibility, *Bact. coli* was grown in a vitamin B_{12}-free medium supplemented either with cyanocobalamin or with an extract of calf faeces. The cells were harvested by centrifugation and extracted and their vitamin B_{12} activity was measured with *Bact. coli* and with *Lb. leichmannii*.

The ratios of activities in Table 4 show that the initial discrepancy between *Bact. coli* and *Lb. leichmannii* activities of the faeces extract persisted into the extracts of the *Bact. coli* cells.

Table 4. *Effect of growing* Bacterium coli *in basal medium with cyanocobalamin, and in basal medium with calf-faeces extract, on vitamin B_{12} activity for* Bact. coli *and* Lactobacillus leichmannii. *Cells harvested and cyanide-extracted before assay*

Solution assayed	Ratio, vitamin B_{12} activity for *Bact. coli* : vitamin B_{12} activity for *Lb. leichmannii*
Cyanocobalamin	1·0
Extract of *Bact. coli* cells, grown with cyanocobalamin	1·1
Calf-faeces extract	6·2
Extract of *Bact. coli* cells, grown with calf-faeces extract	4·5

Chick assays

The results of chick assays of the vitamin B_{12} activity of samples of dried rumen contents and dried calf faeces are listed in Table 5, together with results of microbiological assays of the same materials.

Table 5. *Comparison of chick and microbiological assays of the vitamin B_{12} activity** (μg/g) *of dried calf faeces and rumen contents*

Substance	Chick assay	Microbiological assay: With *Bact. coli*	Microbiological assay: With *Euglena gracilis*	Microbiological assay: With *Lb. leichmannii*
Dried rumen contents	0·2 (0·1–0·5)†	3·8	3·6	1·1
Dried faeces	0·4 (0–0·8)†	10·6	—	1·9

* Expressed as cyanocobalamin.
† True fiducial limits at $P=0·95$.

An interesting finding, as yet unexplained, was that the apparent vitamin B_{12} activity of dried calf faeces for chicks increased markedly when crystalline cyanocobalamin was included in the test diets (Table 6).

Effect of incubation on vitamin B_{12} activity of calf faeces

The results in Table 7 show that incubation of freshly voided calf faeces for 3 days at 30° caused a marked increase in their vitamin B_{12} activity for chicks, whereas their activity for *Lb. leichmannii* showed little or no change and that for *Bact. coli*

diminished. This fall in *Bact. coli* activity during incubation was subsequently confirmed with several different samples, the *Lb. leichmannii* activity again showing little or no change.

Table 6. *Vitamin B_{12} activity for chicks of calf faeces given with and without cyanocobalamin*

Substance	Apparent vitamin B_{12} activity* (μg/g) Result	True fiducial limits at $P=0.95$
Dried calf faeces	0·4	0–0·7
Dried calf faeces with 1 μg cyanocobalamin (added before drying)	3·6	1·5–7·0
Dried calf faeces with 1 μg cyanocobalamin (added after drying)	3·1	0·8–5·5

* Expressed as cyanocobalamin.

Table 7. *Effect of incubation on vitamin B_{12} potency* (μg/g) of calf faeces*

Substance	Chick assay	True fiducial limits at $P=0.95$	Microbiological assay With *Bact. coli*	With *Lb. leichmannii*
Fresh faeces	0·29	(0·02–0·7)	13	1·8
Incubated faeces	0·64	(0·04–1·4)	6·2	1·7

* Expressed as cyanocobalamin.

DISCUSSION

We were surprised to find that *Lb. leichmannii* and *Bact. coli* gave such widely discrepant results in assays of gut contents and faeces, and especially that this difference persisted in extracts of *Bact. coli* cells grown with calf faeces, since the two methods had been found earlier to agree well for a number of other materials, such as crude and refined liver extracts and fish solubles (Table 8). However, the range of natural

Table 8. *Vitamin B_{12} activity of some substances for* Bacterium coli *and* Lactobacillus leichmannii

Substance	Vitamin B_{12} activity* (μg/g) Assayed with *Bact. coli*	Assayed with *Lb. leichmannii*
Crude liver extract	2·4	2·5
Refined liver extract	81	78
Condensed fish solubles	0·26	0·39
Whale-meat meal	0·08	0·11
White fish meal	0·05	0·05

* Expressed as cyanocobalamin.

materials that can be assayed by both methods is limited by the low sensitivity of the *Bact. coli* cup-plate assay.

The low values obtained by chick assay were surprising in the light of our normal experience that with crude natural materials, such as fish solubles and liver extracts,

the chick test gives higher values than the microbiological (cf. Coates, Harrison & Kon, 1951). These low values were apparently not due to any depression of the growth of the chicks by the materials tested. In assays of riboflavin, nicotinic acid, vitamin B_6 and pantothenic acid, dried rumen contents were given at higher levels without growth inhibition, and the results of these tests were in excellent agreement with those obtained by microbiological and chemical assays (Coates, Ford, Harrison, Kon, Shepheard & Wilby, 1952).

McGinnis, Stevens & Groves (1947) and Groschke, Thorburn, Luecke, Thorp & McMillen (1950) have shown that the vitamin B_{12} activity for chicks of the faeces of hens and pigs increases on incubation. Our finding that the vitamin B_{12} potency of calf faeces for chicks increases on incubation, whereas the activity for *Lb. leichmannii* shows little or no change, suggests that during incubation a form of vitamin B_{12} active for *Lb. leichmannii* acquires activity for chicks. This finding will be more fully discussed in a subsequent paper.

We interpret our findings as indicating the presence in gut contents and faeces of a vitamin B_{12}-active substance (or substances) that cannot be converted into vitamin B_{12} by treatment with cyanide, but is apparently differently active for chicks and the several assay micro-organisms.

Cyanide clearly facilitates extraction of these active substances, possibly in the same way as it assists the extraction of cobalamins from liver, where it apparently releases them from bound forms (Wijmenga, Veer & Lens, 1950–1).

These findings raise again the problem of specificity in test procedures and of the measurement as cobalamin of other related substances active for certain test organisms.

SUMMARY

1. The vitamin B_{12} activity of gut contents and faeces for *Bact. coli* and *Euglena gracilis* was considerably higher than that for *Lb. leichmannii*. The assay procedures satisfied the accepted criteria of validity and were adjudged capable of measuring vitamin B_{12} in these materials.

2. Much higher results for all three test organisms were obtained by extraction in the presence of cyanide, but the discrepancies between the results with the different organisms remained.

3. Assays with chicks gave markedly lower results than any of the microbiological tests. However, incubation of fresh calf faeces caused an increase in the vitamin B_{12} potency measured by chicks, but no increase in that measured by micro-organisms.

4. We interpret our results as indicating the presence in gut contents and faeces of vitamin B_{12}-like compounds not convertible to cyanocobalamin by cyanide and having different activities for chicks and for several test micro-organisms.

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Vitamin B_{12}-like Compounds

2. Some Properties of Compounds Isolated from Bovine Gut Contents and Faeces*

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We have shown (Coates, Ford, Harrison, Kon & Porter, 1953) that different micro-organisms widely used for determining vitamin B_{12} give markedly different responses to the vitamin B_{12} activity of extracts of faeces and gut contents prepared in the presence of cyanide. We postulated that such materials contained a vitamin B_{12}-like substance or substances not readily convertible into cyanocobalamin by treatment with cyanide, and differently active for chicks and for different test micro-organisms.

In this paper we show that gut contents and faeces contain at least three such compounds having different physical and biological properties. The compounds were initially separated by chromatography on filter-paper and demonstrated bio-autographically (cf. Cuthbertson & Smith, 1949; Winsten & Eigen, 1948). Subsequently partition chromatography on kieselguhr was used to prepare larger amounts of them.

EXPERIMENTAL AND RESULTS

Microbiological assays

The methods used were described by Coates *et al.* (1953). In addition, *Bacterium coli* was used in the tube-assay technique described by Burkholder (1951), except that the basal medium was modified by substituting thiomalic for thioglycollic acid.

Paper chromatography

Preparation of extracts

Rumen contents or faeces were extracted in the presence of cyanide at pH 5, either by steaming with water or by heating under reflux with 75% aqueous acetone. The water extracts were clarified by centrifugation. The acetone extracts were filtered, evaporated under reduced pressure to remove acetone and centrifuged.

* Read in part before the Biochemical Society (Ford, Kon & Porter, 1951; Ford & Porter, 1952).