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Vitamin B_{12}-like Compounds

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

By J. E. FORD and J. W. G. PORTER
National Institute for Research in Dairying, University of Reading

(*Received* 20 *March* 1953)

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).

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Cyanocobalamin can be adsorbed from aqueous solutions on to charcoal and eluted therefrom with 75% acetone. The above extracts were further purified by such treatment with charcoal; vitamin B_{12} activity was eluted from the charcoal with hot 75% acetone containing 0·01% sodium cyanide and the eluate was evaporated under reduced pressure to give a concentrated solution for chromatography or assay.

Chromatography

The extracts were applied to sheets of Whatman no. 1 paper and chromatographed at 37° for 20 h by the descending technique with water-saturated *sec*-butanol containing a trace of sodium cyanide. The dried paper sheets were applied to plates of seeded *Bact. coli* basal medium. After incubation three distinct zones of vitamin B_{12} activity were visible.

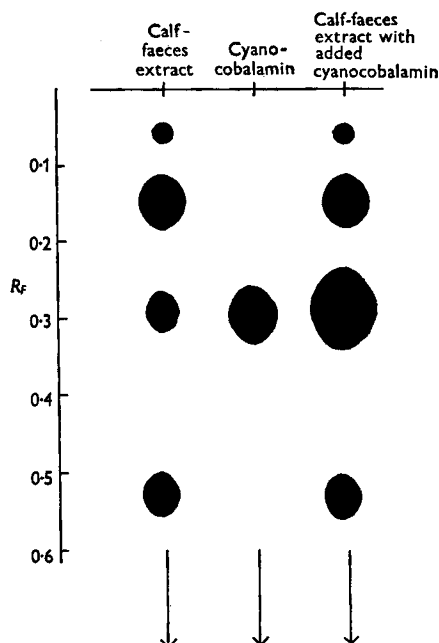

Fig. 1. Bio-autographs with *Bact. coli* of a 'cyanide' extract of calf faeces, with and without added cyanocobalamin

The first zone, fraction A, R_F 0·13, moved more slowly than cyanocobalamin; the second, R_F 0·29, moved at the same rate as, and was inseparable from, cyanocobalamin; and the third, fraction B, R_F 0·54, was faster-moving. We later found that many faecal extracts contained a fourth vitamin B_{12}-active component, fraction C, R_F 0·05, moving even more slowly than fraction A (Fig. 1).

The microbiological activities of the four fractions for various test organisms are indicated in comparative bio-autographs of faecal extracts made with *Bact. coli*, *Lactobacillus leichmannii* and *Euglena gracilis* (Fig. 2). The histograms with *Lb. leichmannii* and *Euglena gracilis* were obtained by cutting the chromatogram into transverse strips and assaying each strip in tube assay. Fig. 2 shows clearly that fraction A and

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the compound presumed to represent cyanocobalamin were active for all three test organisms, whereas fraction B was active only for *Bact. coli* and fraction C had no detectable activity for *Lb. leichmannii* and *Euglena gracilis* when present in the relatively small amounts indicated by *Bact. coli.*

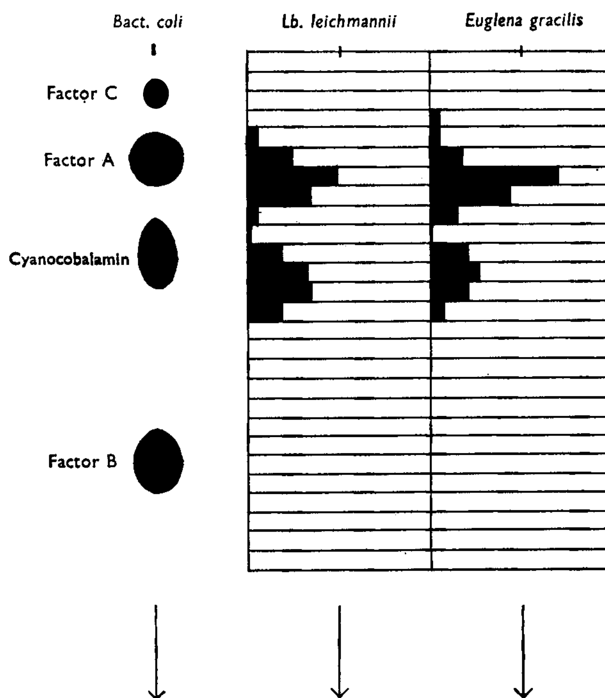

Fig. 2. Comparative bio-autographs with *Bact. coli*, *Lb. leichmannii* and *Euglena gracilis* of a 'cyanide' extract of calf faeces (see above).

Preparation of fractions A and B and cyanocobalamin

Freshly voided faeces from ruminating yearling calves were collected. The faeces were mixed with an equal weight of ethanol and dried in a current of air at 45°. They were then finely milled and treated as outlined in the flow sheet (Fig. 3).

The dried faeces were heated under reflux for 1 h with 50% (v/v) aqueous acetone containing 0·01% sodium cyanide and then filtered, and the residue was washed with hot aqueous acetone. The combined filtrate and washings were evaporated under reduced pressure to remove the acetone. The sediment that appeared during the concentration was centrifuged down and rejected. The supernatant fluid was stirred with charcoal (Sutcliffe and Speakman, no. 5) for 30 min. The charcoal was collected by centrifuging and washed with 5% (w/v) phenol, which removed much coloured impurity. The charcoal was then extracted twice with boiling 65% aqueous acetone containing 0·01% of cyanide. The extract, which was red-brown in colour, was concentrated under reduced pressure to a small volume and poured on to a column of acid-washed alumina (Aluminium Oxide for Chromatographic Adsorption Analysis, British Drug Houses) at pH 3·0. Most of the brown impurities remained at the top of the column

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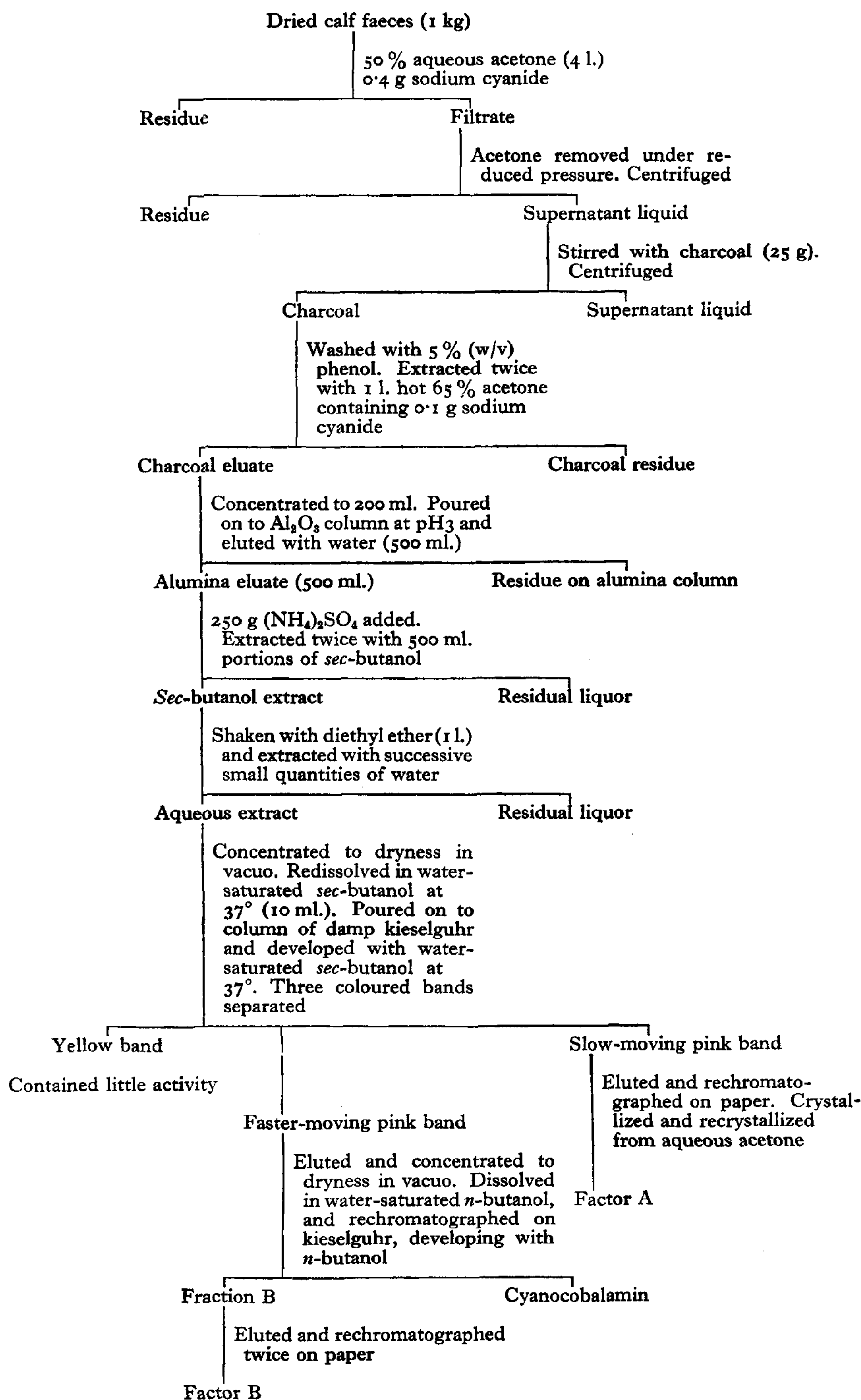

Fig. 3. Flow sheet of the procedure for isolating factors A and B and cyanocobalamin from calf faeces.

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and the effluent was yellow in colour. The column was eluted with water until the runnings were almost colourless and contained little vitamin B_{12} activity, as judged with *Bact. coli*. Quantitative estimates of the yields up to this stage were difficult, but it was clear that as much as 50% of the original activity was lost during the adsorption and elution from charcoal, and a further 10–20% was lost during the chromatography on alumina.

Ammonium sulphate was added to the eluate from alumina, and the solution was extracted several times with *sec*-butanol. Diethyl ether was added to the combined *sec*-butanol extracts and the whole was extracted with successive small volumes of water. The aqueous extract was evaporated to dryness in vacuo and the residue was redissolved in the minimum amount of water-saturated *sec*-butanol containing a trace of cyanide. This solution was carefully poured on to a column of damp kieselguhr, which was developed at 37° with the same solvent. Development resolved the charge into three bands, which were collected separately: the fastest running was yellow and contained little vitamin B_{12} activity; the second band was pink and subsequently resolved into cyanocobalamin and fraction B; the slowest was more intensely pink and yielded only fraction A.

The fraction A was concentrated and twice purified by rechromatography on thick filter-paper (Whatman 3 MM) with the same solvent system. Finally, the band of apparently homogeneous fraction A was cut from the paper and dissolved in a few drops of water; a little acetone was added and the whole allowed to stand in a desiccator over anhydrous acetone until crystals formed. After recrystallization from aqueous acetone the clusters of needle-like crystals appeared homogeneous (but see p. 335). We call this purified crystalline material factor A. The overall yield of crystalline factor A from 1 kg of dried calf faeces varied from 100 to 300 μg.

The second band from the kieselguhr column, consisting of cyanocobalamin and fraction B, was taken to dryness under reduced pressure and then dissolved in water-saturated *n*-butanol containing a trace of cyanide. This solution was poured on to kieselguhr; development with water-saturated *n*-butanol containing cyanide separated a fast-moving pink band of fraction B from a slower-moving band of cyanocobalamin. The yield of fraction B was very small; after further purification 10–30 μg/kg dried faeces of chromatographically homogeneous material were obtained, as estimated spectrographically. Several preparations of fraction B were combined, but the factor could not be crystallized. We call this chromatographically homogeneous material factor B.

The yield of cyanocobalamin also was small (*c.* 50 μg/kg faeces).

Properties of factor A

Physical and chemical

The absorption spectrum of an aqueous solution of the crystalline factor A is shown in Fig. 4(*A*); it closely resembles that of cyanocobalamin, with peaks at 280, 320, 361, 520 and 548 mμ. The addition of excess cyanide caused the colour to change from pink to purple. The absorption spectrum of this solution is shown in Fig. 4(*B*); the peaks are at 278, 305, 368, 540 and 578 mμ, and the only noteworthy difference

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in form from that of the cyanocobalamin cyano-adduct is the absence of inflexion at 288 mμ. The $E^{1\%}_{1\,cm.}$ values for factor A were lower than those for cyanocobalamin. This was probably in part due to hydration, since air-dried material was used.

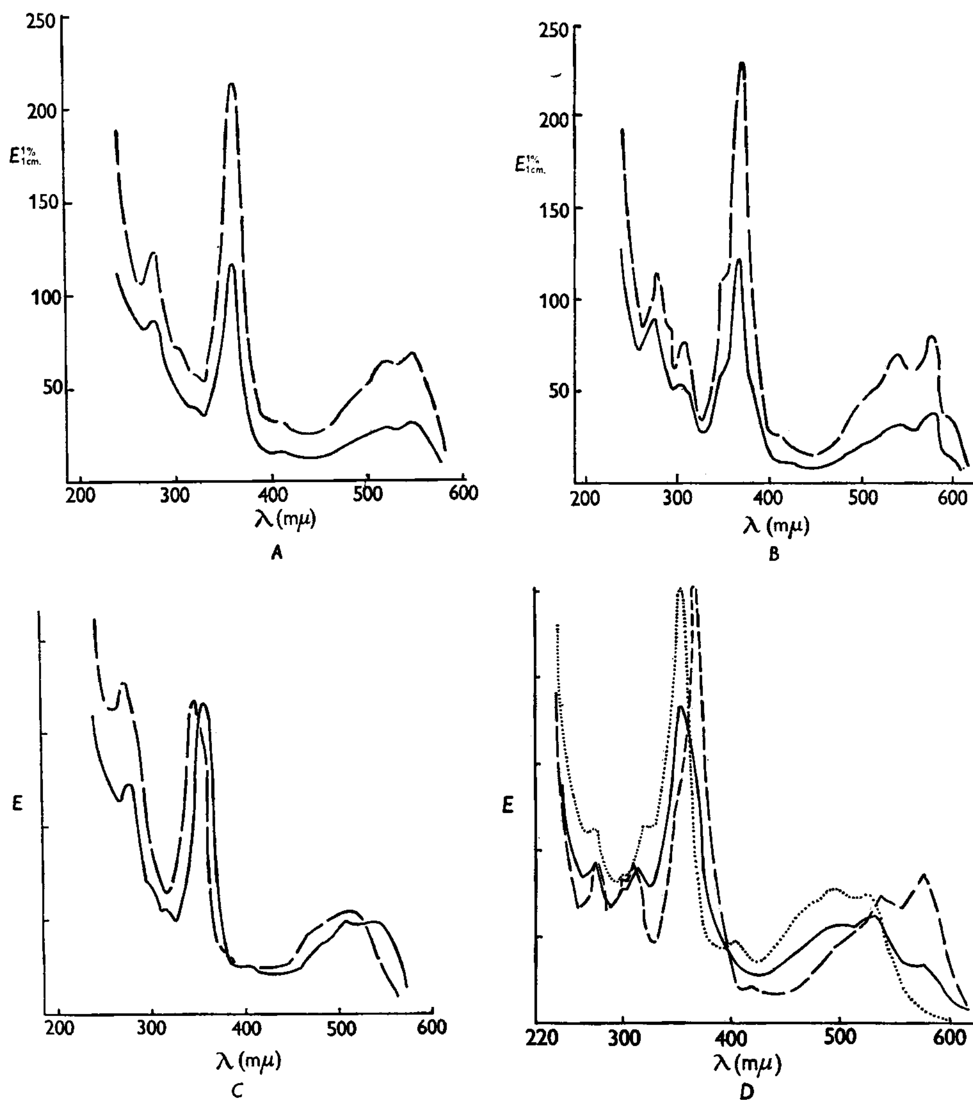

Fig. 4. Absorption spectra of (*A*) factor A (——) and of cyanocobalamin (– – –); (*B*) cyano-adducts of factor A (——) and of cyanocobalamin (– – –); (*C*) factor A before (——) and after (– – –) exposure to light at pH 5; (*D*) factor B (——) and of its cyano-adduct (– – –) and hydroxy-derivative (······).

Factor A also resembled cyanocobalamin in that on passing nitrogen through a warm solution made by dissolving crystalline material in water at pH 5 and in strong light (cf. Boxer & Rickards, 1951) cyanide was evolved. The resulting solution, presumably of the hydroxy compound, had the absorption spectrum shown in Fig. 4(*C*). On addition of cyanide, factor A was reformed.

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Microbiological

The microbiological potencies for *Bact. coli*, *Lb. leichmannii* and *Euglena gracilis* are given in Table 1. Factor A showed activity approaching that of cyanocobalamin in the *Bact. coli* plate and *Euglena* assays, but in tube assays both with *Bact. coli* and with *Lb. leichmannii* its activity was markedly lower.

Table 1. *Relative vitamin B_{12} activities* (µg/g) of factor A and factor B measured by* Bact. coli, Lb. leichmannii *and* Euglena gracilis

Substance	Concentration by weight	*Bact. coli* plate assay	*Bact. coli* tube assay	*Lb. leichmannii* tube assay	*Euglena gracilis* assay
Cyanocobalamin	100	100	100	100	100
Factor A	100	84	32	26·5	80
Factor B	Not known	100	13	0·04	0·03

* Expressed as cyanocobalamin.

Properties of factor B

Physical and chemical

The absorption spectra of solutions of factor B and of its cyano-adduct are shown in Fig. 4(*D*). The peaks are at 276, 315, 355, 503 and 530 mµ, and those for the cyano-adduct are at 276, 310, 367, 540 and 580 mµ.

Factor B readily loses cyanide; when a stream of air was passed through its solution at pH 5 the resulting hydroxy derivative showed peaks at 274, 302, 320, 355, 495 and 525 mµ (Fig. 4(*D*)).

Microbiological

Factor B is active for *Bact. coli*, more so in the cup-plate assay than in the tube assay. It is relatively inactive for *Lb. leichmannii* and *Euglena gracilis* (Table 1).

Factor C

We know little as yet of the properties of factor C. It occurs in cyanide extracts of gut contents and faeces and has been detected in extracts of cells of *Bact. coli*. Factor C appears to have microbiological activities similar to those of factor B in that it is highly active in the *Bact. coli* plate assay and much less so in tube assays with *Bact. coli*, *Lb. leichmannii* or *Euglena gracilis*.

Utilization of cyanocobalamin and of the related compounds by Bact. coli

Bact. coli was grown in the vitamin B_{12}-free basal medium supplemented with factor A or B or cyanocobalamin (we had insufficient factor C to allow its use in such experiments). The cells were harvested by centrifugation and extracted in the presence of cyanide. The extracts were chromatographed and examined by the bio-autographic technique.

Factor A and cyanocobalamin were largely unchanged after this passage through *Bact. coli*, although traces of factor C were detected in the cell extracts.

Factor B could not be recovered after such passage. In its place the cells

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contained a vitamin B_{12}-active compound that appeared from its chromatographic and microbiological properties to be factor C. In some experiments factor C was accompanied by appreciable amounts of factor A and cyanocobalamin. These findings are illustrated in Fig. 5.

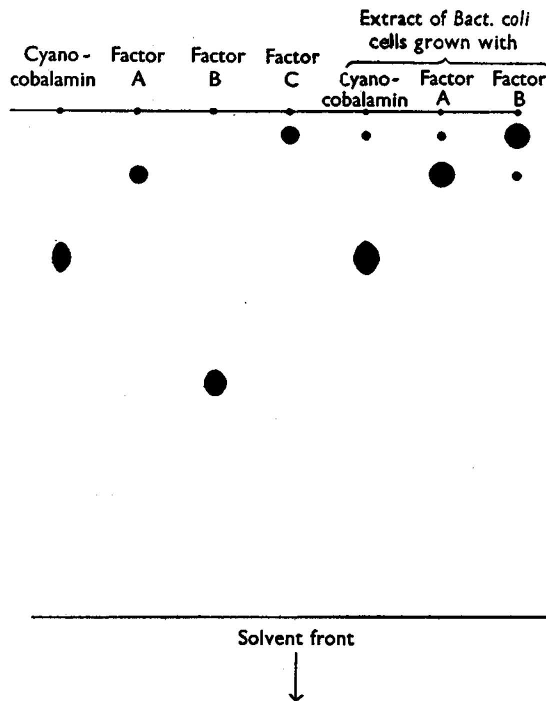

Fig. 5. Bio-autographs with *Bact. coli* of cyanocobalamin and factors A, B and C, and of extracts of *Bact. coli* grown with cyanocobalamin, factor A or factor B.

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

An earlier unexplained finding (Coates *et al.* 1953) was that incubation of freshly voided calf faeces caused a marked increase in their vitamin B_{12} potency for chicks, whereas their activity for *Lb. leichmannii* showed little or no change and that for *Bact. coli* diminished. This problem was now further examined using the bio-autographic technique.

Samples of freshly voided calf faeces were each divided into two portions: one was incubated at 30° for 3 days, while the other was stored at 0°. Equal portions of extracts representing equal weights of the incubated and unincubated faeces were chromatographed on paper. The chromatograms were cut transversely into strips, and the vitamin B_{12} activity of each strip was measured with *Lb. leichmannii.* It is evident from Fig. 6 that incubation resulted in a marked increase in the amount of cyanocobalamin and in a decrease in the amount of factor A. It should be noted that the cyanocobalamin content increased, whereas the total vitamin B_{12} activity showed little change. This finding is being further investigated.

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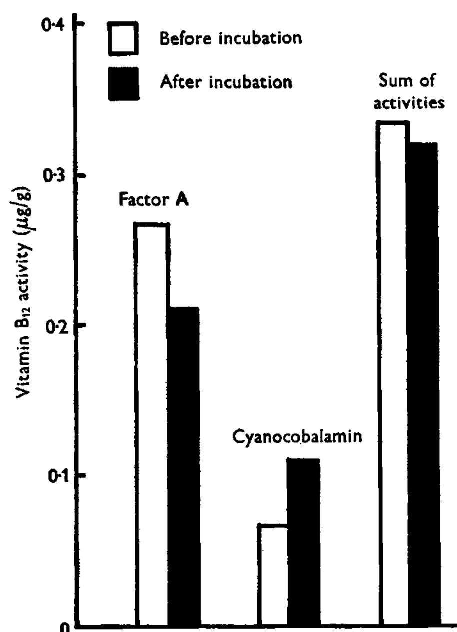

Fig. 6. Changes in the amounts of factor A and cyanocobalamin resulting from the incubation of calf faeces. Activities were measured with *Lb. leichmannii* (see p. 333).

DISCUSSION

It is apparent that the vitamin B_{12} activity for *Bact. coli* of calf gut contents and faeces is contributed by at least four compounds: factor A, factor B, factor C and cyanocobalamin. Factors A, B and C closely resemble cyanocobalamin in certain physical and chemical properties; thus we showed that factors A and B form hydroxo-compounds and cyano-adducts and may well each form a series of compounds analogous to the cobalamins.

The absorption spectrum of factor A closely resembles that of cyanocobalamin. The spectra of the cyano-adducts are also similar, but that of factor A has no inflexion at 288 mμ. There is strong evidence that this inflexion in the spectrum of the cyano-adduct of cyanocobalamin is associated with the presence in the molecule of the benziminazole group (Beaven, Holiday, Johnson, Ellis, Mamalis, Petrow & Sturgeon, 1949; Beaven & Holiday, 1952), and it seems possible, therefore, that this group is absent from factor A.

Hausmann (1949) and Hausmann & Mulli (1952) have reported the isolation of cobalt-containing pigments from cow and sheep faeces and other sources. They consider these substances to be vitamin B_{12}-peptide conjugates; they were highly active microbiologically, but had little or no anti-pernicious anaemia activity unless they were treated with proteolytic enzymes or cyanide. Other workers have also reported the existence of bound forms of vitamin B_{12} from which the free vitamin may be liberated by proteolysis or treatment with cyanide (cf. Smith, 1950–1; Wijmenga, Veer & Lens, 1950–1; Gregory, Ford & Kon, 1952). There is no evidence that factors A, B or C are convertible into cyanocobalamin by any such means.

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Each of the factors is differently active for the several test organisms. In addition, each has different activities relative to vitamin B_{12} for the individual test organisms used in different assay techniques, e.g. *Bact. coli* tube and cup-plate assays (cf. Ford, 1953). Thus the presence of these vitamin B_{12}-like compounds in extracts of gut contents and faeces greatly complicates the measurement of cyanocobalamin itself, and the limitations of the available microbiological techniques must be constantly borne in mind, since these factors may prove to be widely distributed in natural materials, particularly in those subjected to microbial fermentation, e.g. silage. It is clear that chromatography is a useful adjunct to the use of such non-specific techniques for the measurement of cyanocobalamin (but see below). The use of *Bact. coli* in bio-autographic techniques offers a ready means of detecting any substances related to cyanocobalamin that may be encountered in cyanide-treated extracts of natural materials. We have used such a technique to examine the changes during incubation in the vitamin B_{12} potencies of calf faeces. It is evident from the findings illustrated in Fig. 6 that the enhanced activity for chicks can be explained by the increase in the amount of cyanocobalamin. It may well be that cyanocobalamin is formed during incubation at the expense of other factors. Whatever the origin of the cyanocobalamin, this demonstration of its formation during incubation supports the findings of Groschke, Thorburn, Luecke, Thorp & McMillen (1950) and McGinnis, Stevens & Groves (1947).

Factor A is the most abundant vitamin B_{12}-like compound in gut contents and faeces. Factors B and C and cyanocobalamin are present in relatively smaller amounts. Since cyanocobalamin is the major vitamin B_{12}-active constituent of the tissues of animals, including ruminants, it seems possible that of these vitamin B_{12}-like compounds only vitamin B_{12} itself is utilized by the animal; factors A, B and C may be primarily concerned in the metabolism of the gut micro-organisms. We can conclude that cobalt is necessary in ruminant nutrition as a substrate not only for the synthesis of cyanocobalamin essential for the host but also to allow the microbial synthesis of these other vitamin B_{12}-related compounds necessary for the maintenance of the normal gut flora (Ford, Kon & Porter, 1952).

In addition to the vitamin B_{12}-like compounds described here, several other similar compounds have been reported. Wijmenga (1951) isolated from pig faeces a substance he called vitamin B_{12m}; workers at Glaxo Laboratories (Smith, private communication) have isolated from a fermentation liquor a factor, to our knowledge identical with factor B; Pfiffner, Calkins, Peterson, Bird, McGlohon & Stipek (1951) isolated pseudovitamins B_{12} and B_{12b} from cultures of a rumen anaerobe; Lewis, Tappan & Elvehjem (1952) reported the presence in rat faeces of vitamin B_{12f}, and Hausmann (1949) and Hausmann & Mulli (1952) have described further compounds.

In further studies (Ford, 1953; Holdsworth, 1953; Ford, Holdsworth, Kon & Porter, 1953) we have shown that on ionophoresis the factor A described in this paper proved to be not entirely homogeneous but to contain small amounts of other factors. It has now become evident that chromatography and recrystallization are not sufficiently selective to enable the complete resolution of these vitamin B_{12}-like compounds. At the same time we have shown that vitamin B_{12m} (Wijmenga, 1951) also

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was not homogeneous but contained the same active compounds in about the same relative amounts as factor A. Pseudovitamins B_{12} and B_{12b} (Pfiffner *et al.* 1951) also proved impure and each owed its activity to the same major component different from that of factor A (and vitamin B_{12}), and to factor A. We have isolated from rat faeces a 'factor A' that on ionophoresis separated into approximately equal amounts of factor A and pseudovitamin B_{12}, and it seems probable that vitamin B_{12f} (Lewis *et al.* 1952) is such a mixture.

In the light of these findings we consider that the vitamin B_{12} activity for micro-organisms of cyanide extracts of gut contents and faeces is contributed by the following five substances: factor A, factor B, factor C, pseudovitamin B_{12} and cyanocobalamin. A fuller account of the distribution, properties and interrelationships of these compounds will be presented in further papers.

SUMMARY

1. In addition to cyanocobalamin itself three compounds active for *Bact. coli* were found in extracts of gut contents and faeces of calves. These factors, not convertible to cyanocobalamin by cyanide, were named A, B and C.

2. Factor A was obtained from faeces in crystalline form. Factor B was isolated in a chromatographically uniform state, but it could not be crystallized. Factor C was only isolated in minute amount (a few μg).

3. The absorption spectrum of factor A closely resembled that of cyanocobalamin; that of factor B was similar but had different peaks.

4. The vitamin B_{12} activities of the three factors depended both on the test organism used and on the conditions of assay. Factor A was approximately as active as cyanocobalamin for *Bact. coli* in the cup-plate assay and for *Euglena gracilis*, but only about a third as active for *Lb. leichmannii* and *Bact. coli* in tube assays. Factors B and C were highly active for *Bact. coli* in the plate assay, but were relatively inactive in the tube assay and had little or no activity for the other test organisms.

5. Subsequently application of ionophoresis (Ford, 1953; Holdsworth, 1953; Ford *et al.* 1953) showed that the apparently homogeneous factor A described here contained small amounts of the other factors.

6. Incubation of freshly voided calf faeces caused an increase in their content of cyanocobalamin and a decrease in their content of factor A.

7. *Bact. coli* was grown in a vitamin B_{12}-free medium supplemented with factor A or B or cyanocobalamin, and the cells were harvested and 'cyanide'-extracted. Factor A and cyanocobalamin were recovered unchanged, but factor B was transformed into factor C.

8. The possible role of these compounds in animal nutrition is discussed.

We are greatly indebted to Dr E. Lester Smith and Dr S. K. Kon for their interest and advice during the course of this work.

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The Nutrition of the Young Ayrshire Calf

14. Some Effects of Natural and Synthetic Anti-oxidants on the Incidence of Muscular Dystrophy Induced by Cod-liver Oil

By K. L. BLAXTER, F. BROWN and W. A. WOOD
Hannah Dairy Research Institute, Kirkhill, Ayr
and A. M. MacDONALD
University of Glasgow and Royal Hospital for Sick Children, Yorkhill, Glasgow

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The muscular dystrophy of calves caused by giving cod-liver oil (Blaxter, Wood & MacDonald, 1953), the unsaturated acids of cod-liver oil (Blaxter, Brown & MacDonald, 1953*b*) or rations containing lard (Blaxter, Watts & Wood, 1952) may be prevented by giving α-tocopherol.

The mode of action of α-tocopherol in these diseases is unknown. A reasonable supposition, however, is that it acts simply as an anti-oxidant, possibly protecting the animal against the toxicity of unsaturated fatty acids. If this were so, other anti-oxidants might be equally effective. The efficacies of ascorbic acid, ethyl gallate, methylene blue and biotin have therefore been compared with that of α-tocopheryl acetate in preventing the muscular dystrophy induced by giving cod-liver oil to calves.

It has been claimed (Hjärre & Lilleengen, 1936*a*, *b*) that enzootic muscular dystrophy is due to ascorbic-acid deficiency. This is unlikely, for reasons discussed elsewhere (Blaxter & Brown, 1952–3). It is of interest, however, that ascorbic acid is

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