The Microbiological Assay of 'Vitamin B_{12}'. The Specificity of the Requirement of *Ochromonas malhamensis* for Cyanocobalamin

By J. E. FORD
National Institute for Research in Dairying, University of Reading

(*Received* 23 *February* 1953)

It is known that certain natural materials contain substances related to vitamin B_{12}, active as the vitamin for a number of micro-organisms but not for animals: these substances are not in the strict sense cobalamins and cannot be converted into cyanocobalamin by treatment with cyanide. We have studied the identity of several of these vitamin B_{12}-like compounds, isolated by ourselves or by other workers, and concluded that the substances examined were each composed of one or more of five distinct compounds: factors A (vitamin B_{12m}), B and C, cyanocobalamin and pseudovitamin B_{12} (Ford, 1953; Holdsworth, 1953; Ford, Holdsworth, Kon & Porter, 1953). We have further shown that the vitamin B_{12} activities (for micro-organisms) of a variety of natural materials, extracted in the presence of cyanide, were contributed by one or more of these five compounds.

Clearly, the presence in such test extracts of these vitamin B_{12}-like compounds, singly or in combination, can greatly complicate the assessment of cyanocobalamin itself by the available non-specific techniques of assay. There is an evident need for a method of assay capable of measuring cobalamins in the presence of these other related compounds.

Hutner and co-workers (Hamilton, Hutner & Provasoli, 1952; Hutner, Provasoli & Filfus, 1953) have shown that certain chrysomonads require vitamin B_{12} and respond linearly to the vitamin over a wide ratio of concentrations. They drew attention to the potentialities of these phagotrophic protozoa as reagents for high-molecular conjugates of the vitamin and indicated that they might display a specificity for vitamin B_{12} similar to that of birds and mammals.

The present paper describes a method of assay with the protozoan *Ochromonas malhamensis* (Pringsheim isolate) and its application to the measurement of cobalamins in crude extracts and in the presence of vitamin B_{12}-like compounds. The method was developed from that described by Hutner *et al.* (1953). For comparison, tests were also carried out with *Euglena gracilis*, *Bacterium coli* and *Lactobacillus leichmannii*.

EXPERIMENTAL

Tests with Ochromonas

Growth medium. Table 1 shows the composition of the basal medium finally adopted. It was developed empirically by modifying a medium of Hutner, Provasoli & Filfus*

* Private communication from Dr S. H. Hutner. This medium differed in certain respects from a later version described by Hutner *et al.* (1953).

in which, under our test conditions, the organism proved susceptible to non-specific growth stimulants present in certain crude extracts.

Table 1. *Composition of* Ochromonas malhamensis *medium (five times strength)*

Casein hydrolysate* (g)	5	DL-Tryptophane (g)	0·1
Glucose (g)	10	DL-Methionine (g)	0·2
Diammonium hydrogen citrate (g)	0·8	L-Cystine (g)	0·1
KH_2PO_4 (g)	0·3	Choline chloride (mg)	2
$MgSO_4.7H_2O$ (g)	0·2	Inositol (mg)	10
$CaCO_3$ (g)	0·15	*p*-Aminobenzoic acid (mg)	1
'Metals' solution† (ml.)	10	Thiamine (mg)	2
$Na_2MoO_4.2H_2O$ (g)	0·05	Biotin (μg)	10
		Tween 80‡ (ml.)	1

pH adjusted to 5·5. Distilled water to 200 ml.§

* Allen & Hanbury, 'vitamin free'.

† After Hutner (private communication). The solution had the following composition:

Ethylenediamine tetra-acetic acid (g)	5·0	$CoSO_4.7H_2O$ (g)	0·3
$MnSO_4.H_2O$ (g)	6·15	$CuSO_4.5H_2O$ (g)	0·04
$ZnSO_4.7H_2O$ (g)	11	H_3BO_3 (g)	0·06
$FeSO_4.7H_2O$ (g)	1	KI (g)	0·001

Water to 1000 ml.

‡ A polyoxyethylene derivative of sorbitol mono-oleate suitable for use in microbiological cultures. It was kindly given by Messrs Honeywill and Stein Ltd., 21 St James's Square, London.

§ The five-times strength medium was stored in polythene bottles at −20°.

Maintenance of test organism. The test organism was maintained in the basal medium at single strength, supplemented with 0·2 mμg cyanocobalamin/ml. The medium was dispensed in 10 ml. amounts into 50 ml. conical flasks, which were then plugged and sterilized by autoclaving for 15 min at 10 lb. pressure. The organism was transferred in this medium at 5-day intervals, and incubated in a cabinet at approximately 27°, 1 ft. below a 60w. 'striplite' tungsten filament lamp. After 5 days' incubation under these conditions the cell population density in the cultures reached approximately 5,000,000 cells/ml. For inoculum, a 5-day culture was diluted 1 : 10 with sterile, single strength basal medium, and 0·5 ml. was added to each assay tube. Alternatively, and more simply, one drop of the undiluted culture was used to inoculate each assay tube; but in this case greater care was needed to maintain the organisms uniformly in suspension during the process of inoculation.

Assay procedure. Assays were set up in 19 × 150 mm optically matched Pyrex test tubes. A standard solution of cyanocobalamin containing 0·2 mμg/ml. was added to paired tubes at levels of 0·25, 0·5, 1·0, 2·0 and 4·0 ml. Test extracts were added at the same levels, and water was added to the tubes to bring their fluid content to 4 ml. The tubes were then 'dosed' with 1 ml. of the five-times-single-strength basal medium, cotton-plugged and autoclaved for 10 min at 10 lb. pressure. After cooling and inoculating, baskets of tubes were placed in a shaking machine in an incubator at 29° and shaken in darkness for 72 h. The tubes were then steamed, and 5 ml. water were added to each, since 5 (or 5·5) ml. was an inconveniently small volume for measurement in the photometer. Also, growth was otherwise so dense that turbidity readings tended to be at the high end of the scale. Responses were measured turbidimetrically in a Lumetron colorimeter (Photovolt Corporation, New York).

Other things being equal, the rate of growth of the test cultures is governed by the size of the inoculum and the concentration of cyanocobalamin. It is possible to cut down the time needed to carry out the tests, by the use of a heavier inoculum and more potent test solutions. Fig. 1 shows the response to graded amounts of cyanocobalamin after 40 h incubation, the tubes having been inoculated with 0·5 ml. of an

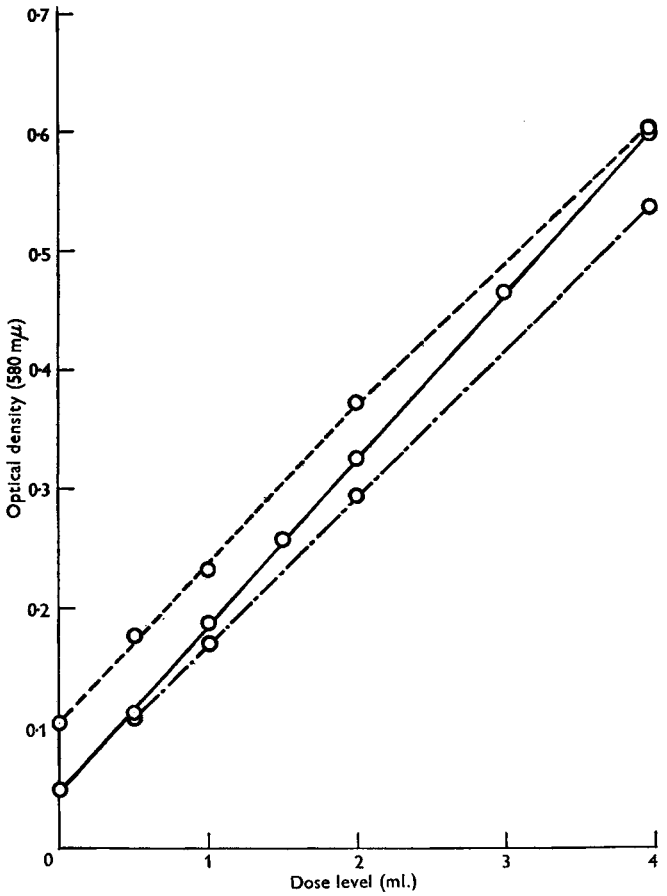

Fig. 1. Effect of size of inoculum and length of incubation on the response of *Ochromonas malhamensis* to graded amounts of cyanocobalamin. ——O——, cyanocobalamin (0·2 mμg/ml.); inoculum, 0·5 ml. of 5-day culture diluted 1 : 10 (see p. 300), incubated 72 h. — - —O— - —, beef-liver extract; inoculum, 0·5 ml. of 5-day culture diluted 1 : 10 (see p. 300), incubated 72 h. – – –O– – –, cyanocobalamin (0·4 mμg/ml.); inoculum, 0·5 ml. of undiluted 5-day culture, incubated 40 h.

undiluted 5-day culture. The figure also shows the response to cyanocobalamin and to an extract of beef liver, after inoculation with 0·5 ml. of the 'normal' diluted inoculum and incubation for 72 h.

Tests with Euglena gracilis. The basal medium used for the *Ochromonas* assays was found to support excellent dark growth of *Euglena*, and tests with this organism were carried out essentially by the technique of the *Ochromonas* assay. A more dilute cyanocobalamin standard was used, containing 0·05 mμg/ml., and the inoculum medium was supplemented with cyanocobalamin at this lower level.

Assays were set up in 50 × 25 mm flat-bottomed specimen tubes covered with aluminium caps. Incubation was carried out for 4 days at 29°, without shaking. Under these conditions growth was as abundant as that obtained when cultures were shaken for the same length of time in plugged 19 × 150 mm test tubes.

Tests with Bact. coli. A mutant of *Bact. coli* requiring cyanocobalamin was used in the tube-assay technique described by Burkholder (1951). The basal medium was modified by substituting thiomalic for thioglycollic acid.

Tests were also carried out by the cup-plate technique (Bessell, Harrison & Lees, 1950; Cuthbertson, Pegler & Lloyd, 1951; Harrison, Lees & Wood, 1951) in the same basal medium as for the tube assays.

Tests with Lb. leichmannii (*ATCC* 4797). With this organism the method and medium of Skeggs, Nepple, Valentik, Huff & Wright (1950) were used, modified only in that the pH of the medium was adjusted to 5·5. Nucleotides were omitted from the medium: baskets of filled tubes were steamed for 30 min before inoculation and incubated for 60 h at 35°. (See Coates, Ford, Harrison, Kon & Porter, 1953.)

Preparation of test extracts. The vitamin B_{12}-like compounds examined, factors A, B and C and pseudovitamin B_{12}, were purified by repeated ionophoresis with 0·5 N-acetic acid (Holdsworth, 1953) and assayed over a wide range of concentration.

The various natural materials examined were extracted by steaming with water containing a trace of sodium cyanide in solution, after adjusting the pH to 5·0 with hydrochloric acid. These extracts were filtered and diluted as appropriate for assay. This method was adopted when it was found that extracts prepared by our accustomed method of steaming with 1% sodium acetate at pH 5·0 in the presence of cyanide would occasionally inhibit growth of *Ochromonas*. It was established that the presence of more than 0·1% sodium acetate in the growth medium markedly inhibited the growth of this organism.

The use of cyanide in the preparation of test solutions is important for two reasons. Firstly, it has been shown (cf. Ford, 1952) to facilitate the extraction of vitamin B_{12}-active substances from natural materials. Secondly, hydroxocobalamin, a form of cobalamin often present in natural materials, is readily inactivated on being heated with certain bacterial growth media and may thus be lost in the process of measurement. In the presence of cyanide hydroxocobalamin is converted to the stable cyanocobalamin.

On assaying certain liver extracts, diluted for test in the absence of cyanide and using media without cyanide, we found unexpectedly low and non-reproducible results both with *Ochromonas* and with *Bact. coli*. Treatment of these extracts with cyanide* gave markedly higher results; in one instance it increased the potency found with *Ochromonas* from 1·4 to 7·7 μg cyanocobalamin/ml.

As it is clearly necessary to ensure that both reference standard and test preparations contain the same active substance, cyanide should be present in trace amounts in all the assay solutions. It is further desirable that media should contain a trace of

* When present in excess, cyanide may inhibit growth of the test organisms, but within limits the amount of cyanide is not critical, since normally it is largely removed during the extraction process of steaming at pH 5. It is our practice to add, for each gram of test sample, from 1 to 10 mg of sodium cyanide depending on the nature and potency of the substance under test.

free cyanide and that the assay tubes should be protected from strong light after autoclaving.

RESULTS

In Table 2 are shown the vitamin B_{12} activities for different test organisms of the different vitamin B_{12}-like compounds. It will be seen that not only do these compounds act differently on the different test organisms, but that each compound may act differently on the same test organism used in different assay techniques. Table 3 further illustrates this point, showing that the vitamin B_{12} activities of factor A and pseudovitamin B_{12} for *Lb. leichmannii* may depend on the composition of the growth medium.

Table 2. *Relative vitamin B_{12}-activities of different compounds, measured by different assay techniques. Results are expressed in terms of the activities measured by the* Bact. coli *tube assay, given as* 100 *in each instance*

Substance	*Bact. coli* (tube)	*Bact. coli* (cup-plate)	*Lb. leichmannii*	*Euglena gracilis*	*Ochromonas malhamensis*
Cyanocobalamin	100	100	100	100	100
Factor A	100	274	64	137	3·4
Factor B	100	580	<0·3	4·2	0
Factor C	100	8500	14	100	0
Pseudovitamin B_{12}	100	1000	400	800	1·2

Table 3. *The vitamin B_{12} activity for* Lb. leichmannii *of factor A and pseudovitamin B_{12}. Effect of modifying the assay medium by substituting ascorbic acid for thiomalic acid*

Substance	Concentration of test solution (μg/ml.)	Potency measured with *Lb. leichmannii*: Medium with ascorbic acid (μg/ml.)	Potency measured with *Lb. leichmannii*: Medium with thiomalic acid (μg/ml.)
Factor A*	100	88	41
Pseudovitamin B_{12}*	100	104	53

* The 'crude' compounds, not purified by ionophoresis, were used (cf. Ford, 1953; Holdsworth, 1953; Ford, Holdsworth, Kon & Porter, 1953).

Table 4. *Measurement with* Ochromonas malhamensis *of cyanocobalamin in admixture with related compounds*

Components of test solution	(μg/ml.)	Vitamin B_{12} activity, measured with *Ochromonas* (μg/ml.)
Cyanocobalamin	1	
Pseudovitamin B_{12}	1*	
Factor A	1*	1·02
Factor B	1*	
Factor C	1*	

* Judged by *Bact. coli* plate assay.

Table 4 shows the response of *Ochromonas* to mixtures of five vitamin B_{12}-like compounds in known amounts. The response of the organism to cyanocobalamin in

the mixture was not modified by the presence of the related vitamin B_{12}-like compounds, and the technique thus appeared capable of measuring cyanocobalamin mixed with them.

Assay of cyanocobalamin in natural materials. Extracts of a number of natural materials were assayed for vitamin B_{12} activity with both *Bact. coli* and *Ochromonas*. The results are set out in Table 5. The potency of each test extract was compared with that of a solution of cyanocobalamin (external standard). In addition, before carrying out the final dilution step in the preparation of each extract two equal portions were taken, and to one of them was added a known amount of the vitamin.

Table 5. *Vitamin B_{12} activity of some natural materials*

	Assayed with *Ochromonas*			Assayed with *Bact. coli*		
Substance tested	By internal standard (μg/g)	By external standard (μg/g)	Mean (μg/g)	By internal standard (μg/g)	By external standard (μg/g)	Mean (μg/g)
Dried calf faeces	0·39	0·39	0·39	2·8	2·2	2·5
Cow-urine extract*	0·0044	0·0047	0·0046	0·011	0·011	0·011
Cow's milk (bulk sample)	0·0030	0·0032	0·0031	0·0028	0·0027	0·0027
Dehydrated minced beef	0·041	0·046	0·044	0·049	0·045	0·047
Refined beef-liver extract *a*	8·6	8·3	8·5	11·7	15·0	13·4
b	—	7·4	—	—	7·8	—
c	—	13·2	—	—	13·5	—
Fish solubles	0·35	0·34	0·35	0·35	0·35	0·35
Aureomycin residue	1·50	1·56	1·53	2·20	1·80	2·00
Dried meadow fescue silage	0·021	0·029	0·025	0·10	0·135	0·12

* Concentrated 200 times by treatment of the original urine extract with charcoal, and eluting from the charcoal with boiling 70% acetone containing a trace of sodium cyanide.

Both portions were then equally diluted to provide the solutions for assay. By comparing the potencies of these two solutions it was possible to calculate the potency of each extract in terms of an internal standard, without reference to the external standard.

Cyanocobalamin in sow's milk. It is known that in sow's milk cobalamin occurs in a 'bound' form, unavailable to *Lb. leichmannii* (Gregory, Ford & Kon, 1952). It is of interest that this bound form of the vitamin cannot be utilized by *Ochromonas*. For this organism, as for *Lb. leichmannii*, *Bact. coli* and *Euglena gracilis*, the vitamin must be liberated by a preliminary digestion of the milk with cyanide-activated papain. It thus seems that this high-molecular complex of vitamin B_{12} is no better utilized by the particle-ingesting protozoan, *Ochromonas malhamensis*, than by the other test organisms less adapted to utilizing intact proteins.

DISCUSSION

It is clear that results obtained by the use of non-specific techniques in the assay of cyanocobalamin are of limited value, and that application of the usual tests of validity may be insufficient to guarantee the presence of the vitamin. It will be seen from Table 5 that in very few of the tests reported here was there any marked discrepancy

between results obtained with external or with internal standards, with either test organism indicating that cyanocobalamin had the same growth-promoting potency in presence or absence of the other substances present in test extracts. Such agreement between results with both types of standard would normally be accepted as providing some evidence for the validity of the results; but here this criterion is of little value, and it is essential first to ensure that the test organism used is capable of responding specifically to cyanocobalamin.

In a survey of a wide range of natural materials we found (Ford, Holdsworth & Porter, 1953) that, in general, extracts of animal tissues contained preponderantly cyanocobalamin, sometimes accompanied by relatively small amounts of the other vitamin B_{12}-like compounds. On the other hand, in natural materials subjected to bacterial fermentation and in various bacterial species, these other compounds were generally present in larger amounts. We found, as expected, that, when microbiological results with *Bact. coli* and *Ochromonas* were in agreement, the substances examined could be shown to contain mainly cyanocobalamin, whereas when the two test micro-organisms gave widely differing results the difference was due in the main to the presence of relatively large amounts of the other compounds.

Of the selection of results presented in Table 5, those representing the refined beef-liver extract, *a*, are surprising, as in our experience extracts of fresh beef liver contained mainly cyanocobalamin. The fact that certain commercial liver extracts were found to contain relatively large amounts of non-specific vitamin B_{12}-activity for *Bact. coli* may imply that in the processes of manufacture this activity, representing the vitamin B_{12}-like compounds and possibly methionine, increased in amount in relation to cyanocobalamin.

It is evident from Tables 2 and 4 that *Ochromonas* has a specificity of a high order for cyanocobalamin in the sense that, of the vitamin B_{12}-like compounds examined, only factor A and pseudovitamin B_{12} had cyanocobalamin activity for this organism, and that much less than cyanocobalamin itself. Further, it is possible that even this low activity may have been wholly contributed by cyanocobalamin still present as an impurity in the two compounds, since the amounts of the 'crude' compounds initially available were very small and did not permit the repetition of the ionophoretic purification procedure as often as was desirable. Even so, with factor A it is unlikely that such impurity could account for all the activity found, and it is more probable that this substance has an intrinsic slight activity for *Ochromonas*.

Ochromonas, therefore, commends itself as a test organism capable of responding almost specifically to cyanocobalamin, even when in crude extracts and accompanied by other vitamin B_{12}-like compounds.

SUMMARY

1. A method is described of using the protozoan *Ochromonas malhamensis* for the assay of cyanocobalamin.

2. A number of vitamin B_{12}-like compounds were examined and found unable to replace cyanocobalamin in the nutrition of this organism.

3. Extracts of a number of natural materials were assayed for vitamin B_{12} activity

with *Ochromonas* and with *Bact. coli*. The *Ochromonas* technique was adjudged capable of measuring cyanocobalamin specifically in a variety of crude extracts, some known to contain vitamin B_{12}-like compounds active for *Bact. coli*.

I wish to thank Drs E. S. Holdsworth and S. K. Kon for helpful interest in this work.

REFERENCES

Bessell, C. J., Harrison, E. & Lees, K. A. (1950). *Chem. & Ind.* p. 561.
Burkholder, P. R. (1951). *Science*, **114**, 459.
Coates, M. E., Ford, J. E., Harrison, G. F., Kon, S. K. & Porter, J. W. G. (1953). *Brit. J. Nutr.* **7**, 319.
Cuthbertson, W. F. J., Pegler, H. F. & Lloyd, J. T. (1951). *Analyst*, **76**, 133.
Ford, J. E. (1952). *Brit. J. Nutr.* **6**, 324.
Ford, J. E. (1953). *Nature, Lond.*, **171**, 149.
Ford, J. E., Holdsworth, E. S., Kon, S. K. & Porter, J. W. G. (1953). *Nature, Lond.*, **171**, 150.
Ford, J. E., Holdsworth, E. S. & Porter, J. W. G. (1953). *Proc. Nutr. Soc.* **12**, xi.
Gregory, M. E., Ford, J. E. & Kon, S. K. (1952). *Biochem. J.* **51**, xxix.
Hamilton, L. D., Hutner, S. H. & Provasoli, L. (1952). *Analyst*, **77**, 618.
Harrison, E., Lees, K. A. & Wood, F. (1951). *Analyst*, **76**, 696.
Holdsworth, E. S. (1953). *Nature, Lond.*, **171**, 148.
Hutner, S. H., Provasoli, L. & Filfus, J. (1953). *Ann. N.Y. Acad. Sci.* **55**, 852.
Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W. & Wright, L. D. (1950). *J. biol. Chem.* **184**, 211.

Antibiotics in the Diet of the Fattening Pig

BY R. S. BARBER, R. BRAUDE, S. K. KON AND K. G. MITCHELL
National Institute for Research in Dairying, University of Reading

(*Received* 2 *March* 1953)

The first indication by Stokstad, Jukes, Pierce, Page & Franklin (1949) that the antibiotic, aureomycin, stimulated growth in chicks led to numerous experiments on the effects of adding antibiotics or fermentation residues containing antibiotics to the diet of chicks, pigs and other animals. Braude, Wallace & Cunha (1953), reviewing the greater part of this work with pigs up to the beginning of 1952, emphasized the complexity of the subject and indicated some of the many and varied factors that appear to have influenced results. From the large amount of evidence reviewed, the authors did not feel justified in drawing any other conclusion than that under some conditions of husbandry some of the antibiotics exert a growth-promoting effect on pigs.

Work published subsequently has, in the main, served to confirm this conclusion, and it is not proposed to present in this paper a review of the further extensive literature on the subject, especially as our review on antibiotics as growth promoters, dealing with all classes of livestock, has just appeared (Braude, Kon & Porter, 1953). In this paper we describe the results of four separate experiments on the effects of adding antibiotics to the diet of fattening pigs. Results of Exps. 1 and 2 have been briefly summarized in preliminary publications (Braude, Kon & Mitchell, 1951; Barber, Braude, Kon & Mitchell, 1952).