VITAMIN ASSAY

Microbiological and Chick Assay of Vitamin B_{12} Activity in Feed Supplements and Other Natural Products

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Recent modifications and improvements of the *Ochromonas* and *E. coli* methods, as well as the details of a chick growth method for determining vitamin B_{12}, are presented. On the basis of comparative determinations of vitamin B_{12} activity in 40 samples, it is concluded that pseudocobalamins—i.e., substances which promote the growth of certain microorganisms but not the growth of animals—do not occur commonly to a large extent in vitamin B_{12} feed supplements. The USP *L. leichmannii* method will give reliable results with most vitamin B_{12} feed supplements, but it is advisable to make at least one test with both the *Ochromonas* and chick methods. Certain fermentation materials contain substances which are inhibitory to chick growth, so that the chick assay method cannot be used in such cases.

The isolation of a crystalline form of the anti-pernicious anemia factor from liver extracts (*25*, *28*) led to the proposal (*25*) that the name "vitamin B_{12}" be given to the substance which had been crystallized and which, like concentrated liver extract (*3*, *7*), had "animal-protein factor" or "factor X" activity for chicks and rats (*22*). The substance also promoted the growth of suitable microbiological test organisms such as *L. lactis* Dorner (*26*), *L. leichmannii* (*16*, *31*), and *Euglena gracilis* (*18*).

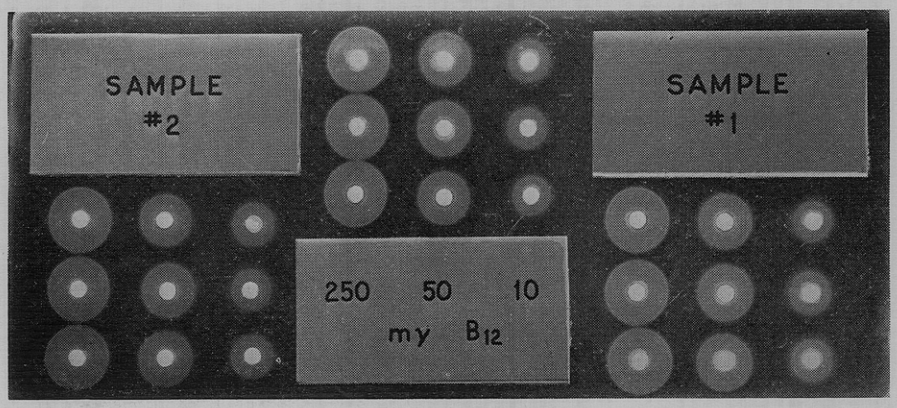
The simplification brought to the field by the new nomenclature was short-lived, for other compounds with equal biological activity were shown to be present in liver extracts and other natural materials (*24*). A fresh attempt to rationalize the terminology was made with the proposal that the compounds be termed "cobalamins," and it was suggested that the original "vitamin B_{12}" be named "cyanocobalamin" in recognition of the presence of the coordinate cyano group which characterized its molecule (*2*). New complexities were soon to appear; a number of other cobalt-containing pigments were shown to be present in natural materials and to differ from the cobalamins in lacking the 5,6-dimethylbenzimidazole group. These other pigments, which may be termed "pseudocobalamins," have successfully eluded a uniform system of classified nomenclature. The first to be partially characterized was "pseudovitamin B_{12}" (*8*, *23*), which contained adenine instead of 5,6-dimethylbenzimidazole. The pseudocobalamins are characteristically inactive for animals and exhibit varying degrees of biological activity in microbiological assays.

Coates and others (*4–6*) have reported that vitamin B_{12} activities of gut contents and feces as determined by *E. coli* and *Euglena gracilis* were higher than those found by *L. leichmannii*, while assays with chicks gave lower results than any of the microbiological tests. Ford, Kon, and Porter (*11*) showed the existence of two pseudocobalamins which they named "factors A and B." Factor B is evidently the pigment which may be obtained by removing the ribotide portion of the vitamin B_{12} molecule by acid hydrolysis (*13*). Ford and Porter in further chromatographic studies found and named factor C (*12*). All three compounds possessed full activity for *E. coli* and various degrees of activity for *L. leichmannii* and *Euglena gracilis*, but were inactive for chick growth. Sjöström (*27*) has reported that half the vitamin B_{12} activity in anaerobically digested municipal sewage sludge is due to factor A, pseudovitamin B_{12}, factor B, factor C, and factor C_2.

The need for an assay method specific for animal activity is obvious, in view of the large amounts (*15*) of vitamin B_{12} activity currently used for various nutritional purposes. Hamilton, Hutner, and Provasoli (*14*) have shown that certain chrysomonads require vitamin B_{12}. One culture, *Poteriochromonas stipitata*, was studied as an assay organism by Barber and others (*1*), who found that fermentation materials showed much lower vitamin B_{12} content by this method than

Figure 1. Pad plate assay of vitamin B_{12} using *E. coli* 11105

Vitamin B_{12} standard is in middle-top area



by conventional methods. Ford (*9*) developed the method further, using *Ochromonas malhamensis* (Pringsheim isolate). All pseudocobalamins which have been detected in natural materials have failed to promote growth of *O. malhamensis* (*17*). Recently additional pseudocobalamins have been produced by growing *E. coli* with various nitrogen bases and factor B (*10*). Some of these are active for *O. malhamensis* but do not occur in natural products. The parallelism between animal and *Ochromonas* activity continues to be valid.

The main purpose of this study was to compare the vitamin B_{12} content of various crude products as determined by three microbiological assay methods and a chick growth method, particularly with regard to the most appropriate use of the USP (*L. leichmannii*) and *Ochromonas* methods for the assay of vitamin B_{12} in nutritional supplements. This communication also presents the detailed procedures for the *E. coli* pad plate method, the *Ochromonas* method, and the chick method as used in these laboratories.

Table I. Basal Medium for *E. coli* Pad Plate Assay Method

Substance	Amounts per Liter of Double Strength, Grams/Liter
Dibasic potassium phosphate	14
Monobasic potassium phosphate	6
Sodium citrate	1
Magnesium sulfate	0.2
Ammonium sulfate	2
Glucose	4
Asparagine	8
	Mg./Liter
Xanthine	20
Arginine	200
Glutamic acid	200
Glycine	200
Histidine	200
Proline	200
DL-Tryptophan	400
Adenine sulfate	20
Guanine hydrochloride	20
Uracil	20
Vitamin solution[a]	

[a] Biotin, 0.1 mg.; pyridoxine, 40 mg.; pyridoxal, 40 mg.; riboflavin, 20 mg.; nicotinic acid, 20 mg.; calcium pantothenate, 20 mg.; thiamine, 20 mg.; folic acid, 10 mg.; *p*-aminobenzoic acid, 10 mg. Vitamins are dissolved in distilled water to a total volume of 100 ml.

Materials and Methods

All samples were extracted for assay by autoclaving 0.5-gram or 1-ml. samples with 25 ml. of pH 4.5 phosphate buffer containing 0.1% sodium metabisulfite (*20*), except that 1% sodium metabisulfite was used with the AOAC samples.

Table II. Composition of Basal Diets

Ingredients	Diet I (Hens), Grams	Diet II (Chicks), Grams
Soybean meal	20	70
Yellow corn meal	57.8	24
Alfalfa meal	5.0	...
Bone meal	4.2	...
Bone ash	...	1
Calcium carbonate	2.3	...
Salt mixture	0.7[a]	1[b]
Sodium chloride	...	0.5
Corn oil + vitamins A, D, and E[c]	...	1.0
Vitamins A and D feeding oil	0.25	...
DL-Methionine	...	0.3
Choline chloride	...	0.2
Procaine penicillin G	...	0.5 mg.
Thiamine HCl	...	1
Riboflavin	1 mg.	1
Niacinamide	5	5
Pyridoxine	1	1
Calcium pantothenate	5	5
Pteroylglutamic acid	1	0.2
Inositol	...	100
Biotin	...	0.02
Vitamin B_{12}	0.0002	...
1-Acetoxy-2-methyl-4-naphthyl sodium phosphate[d]	...	0.2

[a] 67.1% sodium chloride, 4.3% manganese sulfate, 28.6% iodized sodium chloride.
[b] Sodium chloride, 300; dibasic potassium phosphate, 300; monobasic potassium phosphate, 225; magnesium sulfate, 125; manganese sulfate (anhydrous), 25; potassium iodide, 0.3; zinc acetate, 0.7; aluminum sulfate (alunogenite), 0.8 gram.
[c] Vitamin A acetate, 1500 units; vitamin D_3 (Delsterol), 200 AOAC units; mixed tocopherols, 34 mg. dissolved in 1 gram of corn oil (Mazola).
[d] Or any other suitable vitamin K compound.

Four methods of vitamin B_{12} assay were used. The *Escherichia coli* pad plate method uses the vitamin B_{12}–requiring mutant *E. coli*, A.T.C.C. 11105, as described in preliminary reports (*19*, *30*). Paper pads of the type used for antibiotic assays are placed on plates containing the solid assay medium. A satisfactory plate (35 × 16 cm.) made by cementing stainless steel edges to window glass is shown in Figure 1. The inoculum is prepared by transferring 1 or 2 loopfuls of a 24-hour stock culture slant to 10 ml. of sterile (0.85%) saline. The inoculum slant medium consists of Tween 80, 0.05%; glucose, 0.5%; lactose, 0.5%; Difco yeast extract, 1.0%; tryptose (BBL), 0.5%; malt extract, 0.5%; and vitamin-free casamino acids (Difco), 0.5%. The medium is dissolved and filtered, the pH adjusted to 6.5, 1.5% agar added, and the medium autoclaved 10 minutes at 121° C.

To prepare an assay plate, 50 ml. of double-strength basal medium (Table I) is combined with 50 ml. of distilled water, 0.5 ml. of vitamin solution, and 1 gram of agar. Although most of the growth factors present in this medium are not needed by *E. coli*, greater sensitivity for vitamin B_{12} was obtained by their use. The medium is autoclaved 5 minutes at 121° C., cooled to 45° C., and inoculated with 1 ml. of inoculum. The inoculated medium is poured into a carefully leveled plate and allowed to harden. Paper pads, 6.5 mm. in diameter, No. 740-E, available from Carl Schleicher and Schuell, Keene, N. H., are dipped into standard and test solutions, placed on glass plates, and permitted to air-dry for 1 hour before being placed on the inoculated plates.

Three levels of standard and three levels of test sample in triplicate are used, as shown in Figure 1. The standard crystalline vitamin B_{12} solutions are 10, 50, and 250 mγ per ml. Test samples are diluted as nearly as practicable to the same potency as the standards. Zone diameters are measured to the nearest 0.1 mm. A standard curve is drawn on semilogarithm paper, zone diameters being plotted against millimicrograms of vitamin B_{12} per ml. The vitamin B_{12} activity of the test samples is determined by interpolation of the zone diameters on the standard curve.

The *L. leichmannii* USP assay method was that described in the United States Pharmacopoeia (*29*) as modified for the AOAC collaborative study of 1954. Lower blanks were obtained by washing the cells for the inoculum four times instead of once.

The *Ochromonas* method was that described by Ford (*9*) with the following modifications.

Use of 22 × 100 mm. culture tubes.

Heating of all glassware to 200° C. for 4 hours in order to destroy possible traces of residual vitamin B_{12}.

Use of a variable-speed rotary action flask shaker (New Brunswick Scientific Co.) converted to hold culture tubes. The optimum shaking conditions were

found to be a setting of 8, which produced 200 rotations per minute. More severe shaking prevented growth.

The inoculum was centrifuged 4 minutes at 100 r.p.m., the supernatant liquid was removed, and the cells were resuspended in 10 ml. of sterile inoculum medium free of vitamin B_{12}.

Addition of 2 γ of sodium cyanide per ml. of final medium.

Use of the AOAC (*20*) sample extraction procedure.

A typical standard curve is shown in Figure 2.

The chick assay was based on the method described by Jukes and Williams (*19*). The breeding flock at 6 months of age was placed on basal diet I (Table II) and at least 2 weeks elapsed before eggs were used for hatching. Chicks hatched from these eggs were placed in electrically heated battery brooders with $^3/_4$-inch mesh wire floors in an air-conditioned room at 28° C. and fed diet II (Table II) supplemented with the various levels of cyanocobalamin. Duplicate groups of 12 chicks were used for each level of assay. The assay period was 28 days and the average weights at each level were used in constructing a standard response curve to cyanocobalamin, as illustrated in Figure 3.

Results and Discussion

The vitamin B_{12} values obtained by the four methods are shown in Table III. The values are averages of two to four determinations, which agreed within 10% for the microbiological methods and within 30% for the chick method. The chick assay values were in general the lowest and the *E. coli* pad plate values were the highest. In samples 1 through 12 there was a close agreement between the USP and the *Ochromonas* methods. Sample 13, however, was thought from animal studies to contain pseudocobalamins and, as expected, the *L. leichmannii* and *E. coli* methods showed the presence of vitamin B_{12} activity while the *Ochromonas* method did not.

In samples 14 through 34, with a few exceptions, fair agreement was found between the USP and the *Ochromonas* methods. The sewage sludge concentrates, although below the claimed potency, showed similar vitamin B_{12} content by the USP and the *Ochromonas* methods. This differs from Sjöström's (*27*) observation that about half the vitamin B_{12} activity of certain sewage sludges is due to pseudocobalamins. Lillie and others (*21*) obtained vitamin B_{12} values for two sewage sludge preparations which were similar by chick and microbiological assay, and higher microbiological values with three other sludge preparations apparently due to their pseudocobalamin content. The difference may reflect the widely varied microbial action which takes place in the processing of this material.

The values for samples 36 through 40 are the 1954 AOAC collaborative study values obtained in this laboratory. Sample 36 contained 0.5 γ of pseudovitamin B_{12} per ml. and 0.5 γ of cyanocobalamin per ml. The USP and the *E. coli* methods gave values very close to 1 γ per ml., while the *Ochromonas* method determined only the cobalamin.

The values obtained by the chick growth method in general agree with those obtained with the *Ochromonas* method. Samples 14, 15, 16, and 33 were inhibitory to chick growth. The vitamin B_{12} content given in the table for these four samples was calculated from the lowest amount of the sample which promoted growth. The nature of the response made it impossible to assess the biological value accurately. Higher amounts of these samples added to the chick diet gave less growth than this lowest level of sample. If antibiotics were omitted from the chick assay diet, samples containing antibiotics gave a higher apparent vitamin B_{12} content. The addition of an antibiotic to the chick assay diet appeared to eliminate this effect. Penicillin was used in preference to chlortetracycline, because the latter antibiotic is prepared from a fermentation which contains vitamin B_{12}. Lillie and others (*21*) compared vitamin B_{12} assay values obtained by the USP *L. leichmannii* and chick growth methods. Their chick growth method gave vitamin B_{12} values for commercial feed supplements, liver products, and fish solubles which were higher than the microbiological values. However, the basal chick diet was not supplemented with B vitamins and an antibiotic, as was the case in the present investigation.

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Figure 2. Typical standard curve obtained in *Ochromonas* method for vitamin B_{12}

Response of *Ochromonas malhamensis* to vitamin B_{12}. Absorbance determined with Coleman Model 11A spectrophotometer

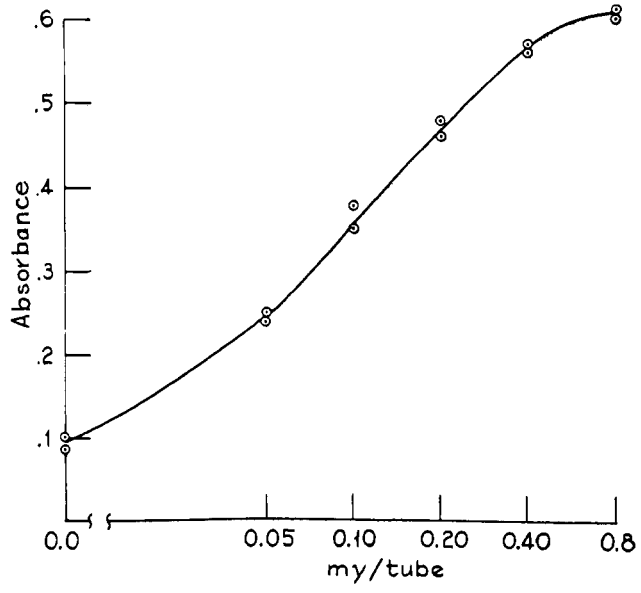

Figure 3. Average weights of groups of 24 chicks at 28 days in response to four amounts of vitamin B_{12}

Table III. Comparison of Vitamin B_{12} Assay Values by Four Different Methods

Sample No.	Type of Product	Stated Content	Assay Results, Mg. Vitamin B_{12}/Lb. USP L. leichmannii	E. coli pad plate	Ochromonas	Chick method
1	Bacterial fermentation	60	29	81	45	31
2	Bacterial fermentation	60	...	99	88	50
3	Bacterial fermentation	70	45	82	59	...
4	Bacterial fermentation	70	41	76	45	44
5	Bacterial fermentation	60	32	73	43	...
6	Bacterial fermentation	80	54	98	82	...
7	Bacterial fermentation	120	96	155	95	64
8	Bacterial fermentation	80	110	140	86	126
9	Bacterial fermentation	399	226	...	238	...
10	Bacterial fermentation	454	499	...	544	...
11	Bacterial fermentation	50	43.2	...	40	...
12	Bacterial fermentation	3.33[a]	2.75[a]	...	2.13[a]	...
13	Pseudovitamin B_{12} fermentation	100	45	78	0	...
14	*Streptomyces* fermentation	20	14	23	23	6 (toxic)
15	*Streptomyces* fermentation	19	12	23	12	0.5 (toxic)
16	*Streptomyces* fermentation	16	14	18	14	4 (toxic)
17	*Streptomyces* fermentation	16	14	17	10	...
18	*Streptomyces* fermentation	12	12	12	13	...
19	*Streptomyces* fermentation	12	10	12	13	...
20	*Streptomyces* fermentation	20	22	30	17	...
21	*Streptomyces* fermentation	6	6	8	4	...
22	*Streptomyces* fermentation	10	9	8	8	5
23	*Streptomyces* fermentation	10	8	1.7	9	10
24	*Streptomyces* fermentation	6	7	7	5	...
25	*Streptomyces* fermentation	12	12	12	13	12
26	*Streptomyces* fermentation	12	10	13	10	12
27	*Streptomyces* fermentation	0.45	0.27	...	0.10	...
28	*Streptomyces* fermentation	0.45	0.36	...	0.31	...
29	*Streptomyces* fermentation	0.45	0.52	...	0.57	...
30	*Streptomyces* fermentation	0.45	0.56	...	0.47	...
31	*Streptomyces* fermentation	...	17	...	17	16
32	Concentrates from sludge	19	13	21	15	25
33	Concentrates from sludge	19	18	13	15	3 (toxic)
34	Concentrates from sludge	16	12	22	15	...
35	Concentrates from sludge	17	14	18	15	15
			γ Vitamin B_{12} per Gram or Ml.			
36	Vitamin B_{12} and pseudovitamin B_{12} solution	1	0.80	0.90	0.56	0.20
37	Vitamin B_{12} concentrate	450	450	454.5	335	190
38	Vitamin B_{12} feed supplement	20	18.1	458	15.6	6.6
39	Fish solubles	0.2	0.20	0.29	0.14	0.20
40	Dried liver	0.9	1.3	2.6	1.1	1.3

[a] Mg. of vitamin B_{12} per ml.

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