

# Microbiological Assay of Vitamin B<sub>12</sub> in the Presence of Tetracycline

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## ABSTRACT

POONAWALLA, F. M. (Alembic Chemical Works, Baroda, India), AND M. R. S. IYENGAR. Microbiological assay of vitamin B<sub>12</sub> in the presence of tetracycline. *Appl. Microbiol.* **13**:755-756. 1965.—Treatment of a pharmaceutical preparation of vitamin B<sub>12</sub> and tetracycline with an ion-exchange resin by the batch method differentially adsorbed tetracycline, making possible a statistically reproducible microbiological assay of vitamin B<sub>12</sub> by the standard *Escherichia coli* plate method.

Present methods (Diding, 1958; Harrison, Lees, and Wood, 1951; Nakazawa et al., 1958) for assay of vitamin B<sub>12</sub> in the presence of antibiotics make use of antibiotic-resistant organisms. Development of resistance in assay organisms has involved the risk of loss of sensitivity in assays, together with slower multiplication rates (Harrison et al., 1951). On the other hand, organisms insensitive to antibiotics, like *Ochromonas malhamensis* (Ford, 1953) or *Euglena gracilis* (Hutner et al., 1949), require several days of incubation before assay.

In the present studies, tetracycline was selectively adsorbed by ion-exchange resins, thus making it possible to assay vitamin B<sub>12</sub> by the usual microbiological assay procedures with antibiotic-sensitive assay organisms.

## MATERIALS AND METHODS

Tetracycline and B<sub>12</sub> were assayed by the four point hole-plate method with *Bacillus cereus* ATCC 11778 (Grove and Randall, 1955) and *Escherichia coli* ATCC 11105 (Harrison et al., 1951), respectively.

The edges of zones of exhibition obtained in assays run as recommended by Harrison et al. (1951) were not sharp enough to permit the zones to be read directly on a Fisher-Lilly Zone Reader. To get sharp and intense zones for this purpose, the procedure was modified as follows: inoculum for assay was prepared by transferring a loopful of the organism from a slant culture to 10 ml of peptone broth (Harrison et al., 1951) and incubating it at 37 C for 24 hr on an incubator-rotary shaker at a speed of 250 rev/min; 1% of broth inoculum was used without washing to inoculate the assay medium.

The assay medium was a modification of that of Davis and Mingioli (1950); 300 ml of each of the following stock solutions were prepared: (i) glucose, 30 g; (ii) K<sub>2</sub>HPO<sub>4</sub>, 21 g; KH<sub>2</sub>PO<sub>4</sub>, 9 g; (iii) sodium citrate, 15 g; (iv) asparagine, 0.6 g;

MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.3 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 3 g; and (v) agar, 10 g. Each solution was prepared and autoclaved separately at 15 psi for 20 min; 100 ml (each) of the first four solutions were mixed aseptically with 600 ml of agar to obtain 1 liter of precipitate-free and colorless medium.

The USP B<sub>12</sub> standard and the samples for assay were dissolved or extracted in citrate-phosphate buffer (pH 4.5) at a level of 1 μg/ml. The buffer was made by dissolving citric acid (1.1 g), Na<sub>2</sub>HPO<sub>4</sub> (1.29 g), and KCN (0.1 g) in 100 ml of distilled water, and was autoclaved at 15 psi for 10 min before use. Necessary dilutions for the assays were made in this buffer without KCN.

Resin-adsorption studies were carried out by the batch method. Vitamin mixtures containing or not containing tetracycline were first autoclaved in the buffer to extract vitamin B<sub>12</sub>, filtered or centrifuged, and then treated with resin. Although in preliminary experiments shorter times were found to be adequate, for the sake of convenience solutions were left in contact with the resins overnight on a rotary shaker at room temperature.

## RESULTS AND DISCUSSION

The results of studies on adsorption of tetracycline and B<sub>12</sub> by various resins are presented in Table 1. The figures represent the quantities of B<sub>12</sub> and tetracycline adsorbed per gram of various resins.

All cationic resins showed very high capacity to adsorb B<sub>12</sub>, and these were considered unsuitable for our purpose. Adsorption of B<sub>12</sub> was poor with both strongly and weakly basic resins. The results indicated that at least four of the anionic resins could be used, depending on the level of interfering tetracycline. Among these, Amberlite IRA-400 (OH<sup>-</sup>), which adsorbed the maximal quantity of tetracycline per unit weight of resin, was considered the most suitable for further studies.

Studies were carried out on recovery of B<sub>12</sub>

TABLE 1. Adsorption of tetracycline and vitamin B<sub>12</sub> by ion-exchange resins\*

Resin	Tetra-cycline (mg/g of resin)	B <sub>12</sub> (μg/g of resin)
Amberlite IR-120 (H <sup>+</sup> ).....	4.5	4.65
Dowex-50W (H <sup>+</sup> ).....	4.5	4.75
Amberlite IRC-50 (H <sup>+</sup> ).....	2.35	4.5
Amberlite IRA-400 (OH <sup>-</sup> ).....	3.95	0.08
Amberlite IR-45 (OH <sup>-</sup> ).....	2.45	0
Amberlite IRA-401 (OH <sup>-</sup> ).....	0.65	1.18
Amberlite IR-4B (OH <sup>-</sup> ).....	1.58	0
Dowex, 1x-8 (Cl <sup>-</sup> ).....	0.58	0.88
Permutit SRA-65 (Cl <sup>-</sup> ).....	0.98	0.08

\* Each resin (2 g) was added to a 10-ml solution of either tetracycline (1 mg/ml) or B<sub>12</sub> (1 μg/ml) in 250-ml flasks and shaken overnight. Adsorption was calculated from residual amounts in solution as determined by plate assay (*B. cereus* for tetracycline and *E. coli* for vitamin B<sub>12</sub>).

from a mixture, in powder form, of vitamins and tetracycline hydrochloride considered as representative of current pharmaceutical preparations of tetracycline containing vitamin B<sub>12</sub> and high concentrations of several other vitamins. This mixture had the following composition: thiamine hydrochloride, 120 mg; riboflavine, 11 mg; pyridoxine hydrochloride, 6 mg; niacinamide, 55 mg; calcium pantothenate, 27.5 mg; ascorbic acid, 165 mg; magnesium stearate, 2.0 mg; cyanocobalamin (B<sub>12</sub>), 7 μg; and tetracycline hydrochloride, 250 mg (when included).

To check the reliability and reproducibility of the resin method, six portions each of 2.224 g (1.224 g of vitamin mixture plus 1 g of tetracycline) of the vitamin-antibiotic mixture were treated and assayed in the same experiment. Each portion was extracted with 28 ml of phosphate-citrate-cyanide buffer by autoclaving at 15 psi for 10 min. It was observed that 55 to 60% of the antibiotic was destroyed on autoclaving. The suspensions were filtered and diluted 100-fold with buffer not containing cyanide; 25 ml of each of the diluted solutions was treated overnight with 1 g of resin. The solution so treated was expected to contain 10 mμg of vitamin B<sub>12</sub> per ml, assuming 100% recovery. This was determined by assaying at two levels, 10 and 5 mμg/ml, against USP B<sub>12</sub> standard.

In a second set of six samples of the same vitamin mixture without the antibiotic, B<sub>12</sub> was extracted by the same procedure except for the omission of the resin treatment, and was assayed. In the presence of the antibiotic, estimates of B<sub>12</sub> made by the resin procedure were equally reproducible and statistically reliable (Table 2).

Where the ratio of tetracycline and B<sub>12</sub> is such

TABLE 2. Recovery by assay of vitamin B<sub>12</sub> in a multivitamin mixture in the presence and absence of tetracycline

Sample	B <sub>12</sub> (no resin)	B <sub>12</sub> + tetracycline (with resin)*
	%	%
1	99.9	95.5
2	94.9	104.5
3	106.4	99.0
4	103.9	108.0
5	93.0	92.5
6	109.0	101.9

\* Overnight adsorption with Amberlite IRA-400 (OH<sup>-</sup>). Standard error, 5.8 (6.3 with B<sub>12</sub> alone); *F* (experimental), 1.23 (tabulated as 3.4 at 10% level); *t* experimental, 0.28 (tabulated, 2.23 at 5% level).

that the quantity of resin required to adsorb tetracycline completely also adsorbs a small percentage of B<sub>12</sub> consistently, it should still be possible to assay B<sub>12</sub> by similarly treating the standard with resin.

It is hoped that this method will be useful not only for pharmaceutical formulations but also for differential assay of B<sub>12</sub> in mixed B<sub>12</sub> and tetracycline fermentations.

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