

## The Assay of Vitamin B<sub>12</sub>

### I. Factors affecting the Response of *Lactobacillus lactis* Dorner ATCC 8000 to Vitamin B<sub>12</sub>

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**SUMMARY:** For maximal zones of 'exhibition' in the plate test for vitamin B<sub>12</sub> with *Lactobacillus lactis* Dorner ATCC 8000, using the media described, the presence of both oxygen and vitamin B<sub>12</sub> is necessary. Under anaerobic conditions the vitamin is not an essential growth factor and under highly aerobic conditions growth does not occur even in its presence. Under the conditions of maintenance used variant strains were not detected.

When grown in the presence of vitamin B<sub>12</sub> the bacteria may store sufficient of the vitamin to render measurements of growth useless for assay purposes: when grown under anaerobic conditions the culture obtained is unsuitable for use as an inoculum. A technique for preparing satisfactory inocula of the test organisms and the conditions necessary for well-defined responses to vitamin B<sub>12</sub> are described.

A cup-plate test for the assay of vitamin B<sub>12</sub> has been described by Cuthbertson (1949), Foster, Lally & Woodruff (1949) and Cuthbertson, Lloyd & Pegler (1951). In this method a suitable agar medium deficient in the factor under investigation is inoculated with the test organism and is poured into Petri dishes; cups cut out of the agar are filled with appropriate dilutions of standard or test solutions. On incubation under suitable conditions, growth of the organism may occur in sharply-defined circular 'zones of exhibition' around the cups. The diameters of these zones were found to be related to the concentration of the added growth factor and may be used to compare the amounts of growth factor present in the test and standard solutions. The method for estimating vitamin B<sub>12</sub> is similar to that for aneurin (Bacharach & Cuthbertson, 1948). In developing working details of the assay, a number of observations were made on factors affecting the growth of *Lactobacillus lactis* Dorner ATCC 8000 and its response to vitamin B<sub>12</sub>; they are reported in this paper.

#### *Media used*

In preliminary work we used a charcoal-treated digest of vitamin-free casein to prepare medium A of the composition shown in Table 1, similar to that of Roberts & Snell (1946). With some batches of medium A the addition of tomato juice was not necessary, whereas with others tomato juice supplied an essential growth factor. We also observed that only a few batches of vitamin-free casein could be used to prepare satisfactory media, but suitable digests could usually be prepared with casein freshly precipitated from skim milk or from reconstituted separated full-cream milk powder. To such digests the addition of tomato juice was unnecessary. Eventually charcoal-treated tryptic digests of reconstituted spray-dried separated milk, or of ether-extracted roller-dried full-cream milk, were found to be as satisfactory as any casein digest tried. Medium B, Table 1, was consequently used in most of this work.

*Effect of reducing agents*

When tryptic digests of casein or milk were stored under toluene at 4° they deteriorated, becoming useless for assay after 1–3 weeks; that is to say, media prepared from these aged digests gave poor zones of growth with pure vitamin B<sub>12</sub>. In some instances no growth occurred at all, even after the addition of vitamin B<sub>12</sub>, but growth zones could be observed with certain crude materials containing vitamin B<sub>12</sub> and other substances. From this it appeared that a growth factor essential present in the fresh casein digest disappeared under

Table 2. *Composition of vitamin B<sub>12</sub>-deficient agar media*

	A	B		A	B
Casein digest (ml.)*	330	—	Adenine (mg.)	10	10
Milk digest (ml.)†	—	330	Guanine (mg.)	10	10
Glucose (g.)	20	20	Uracil (μg.)	10	10
L-Cystine (g.)	0.1	0.1	Lactose (g.)	2	—
Aneurin HCl (μg.)	500	500	Tomato juice (ml.)	50	50
Biotin (μg.)	4	4	Tween (g.)	0.3	0.3
Folic acid (μg.)	2	2	Salt solution C (ml.)‡	20	20
<i>p</i> -Aminobenzoic acid (μg.)	100	100	Ascorbic acid (mg.)	—	1000
Ca D-pantothenate (μg.)	500	500	K <sub>2</sub> HPO <sub>4</sub> (g.)	0.5	0.5
Pyridoxin HCl (μg.)	500	500	KH <sub>2</sub> PO <sub>4</sub>	0.5	0.5
Pyridoxal (μg.)	—	400	Water to (ml.)	1000	1000
Pyridoxamine (μg.)	—	400	pH	6.8	6.8
Nicotinic acid (μg.)	500	500	Agar (N.Z.) (g.)§	15	18
Riboflavin (μg.)	500	500			

\* Prepared according to Roberts & Snell (1946).

† Prepared as follows: make up 240 g. spray-dried skim milk (or 350 g. ether-extracted roller-dried full-cream milk), with 2 l. water; add 600 mg. pancreatin (Morson) and 16 g. NaHCO<sub>3</sub> and a layer of ether and toluene and digest 3 days at 37°. Add 7–10 ml. glacial acetic acid, steam 30 min., add 70 ml. glacial acetic acid and treat with 40 g. charcoal (Sutcliffe, Speakman No. 5) for 30 min. Repeat charcoal treatment. All filtrations may be assisted by use of adequate amounts of kieselguhr.

‡ Salts C: MgSO<sub>4</sub>·7H<sub>2</sub>O, 10 g.; NaCl, 0.5 g.; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g.; MnSO<sub>4</sub>·4H<sub>2</sub>O, 2.0 g.; Water to 250 ml.

§ From Davis Gelatine Ltd., 29–31 Mitre Street, London E.C. 3.

our conditions of storage. An attempt was made to discover sources of this growth factor by a technique similar to the auxanographic method of Pontecorvo (1949). An aged digest was used to prepare a batch of medium A (Table 1). Solutions of vitamin B<sub>12</sub> and substances under investigation were placed in closely adjacent holes on the plates. No growth occurred around the cup containing vitamin B<sub>12</sub> unless the solution in the adjacent cup contained the growth factor missing from this aged digest. In this way urine, gastric juice, plasma and certain yeast extracts were shown to contain the missing growth factor, but none of these substances was found suitable for use in the basal medium without further purification, because they contained other substances that interfered with the test. The type of results obtained is shown in Fig. 1. When thiolacetic acid or ascorbic acid was placed in a cup adjacent to a cup containing vitamin B<sub>12</sub> solution there was a clearly marked zone of bacterial growth in the region into which both the vitamin B<sub>12</sub> and the thiolacetic or ascorbic acid had diffused.

In subsequent experiments with different concentrations of ascorbic acid or thiolacetic acid 1 mg./ml. allowed the aged media to support growth of *L. lactis* in the presence of vitamin B<sub>12</sub>. Ascorbic acid was consequently incorporated in the medium, 1 mg./ml., and the addition of tomato juice was then found unnecessary.

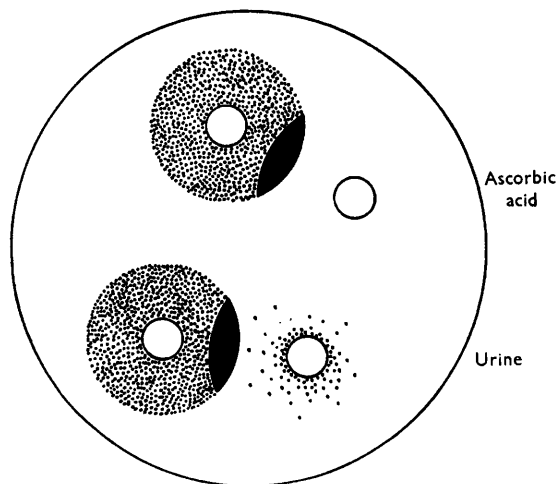


Fig. 1. The effect of reducing agents and urine on response of *Lb. lactis* to vitamin B<sub>12</sub>.

#### *Effect of thickness of the agar in the plates*

A large plate, 15 × 15 in. was poured with 350 ml. of the inoculated agar medium containing ascorbic acid. During the preparation the plate was slightly tilted so that the thickness of the agar varied from 1 to 3 mm. over the breadth of the plate. A solution of vitamin B<sub>12</sub> was placed at various positions on the plate, which was then incubated overnight. The organism responded well to vitamin B<sub>12</sub>, showing clearly defined zones whenever the thickness of the agar was between 1 and 2.5 mm.; where the agar thickness was more than 2.5 mm., growth of the bacteria was unrestricted even in the absence of vitamin B<sub>12</sub>. This effect could be readily repeated in other plates of a similar type. Further, in test-tubes of liquid media containing 0.1 % thiolacetic or ascorbic acid the same unrestricted growth occurred even in the absence of vitamin B<sub>12</sub>. In other plates in which the agar thickness was less than 1 mm. the organism responded little or not at all to vitamin B<sub>12</sub>.

#### *Failure to isolate variant strains*

During the development of the cup-plate method we frequently encountered difficulty with the assay and often suspected that the properties of the strain had changed, especially in view of the report by Shorb & Briggs (1948) that *L. lactis* very readily produces variants. We were unable at any time to demonstrate differences between the culture in use (maintained by weekly transfer in soya-bean medium) and cultures obtained from freeze-dried samples

of the organism. Attempts were made to isolate different strains. On pouring or streaking plates surface colonies were not formed under ordinary aerobic conditions; anaerobically, however, there was good surface growth and colonies could be isolated. No evidence of variation was obtained and we are satisfied that under our conditions of culture this strain is adequately stable.

#### *Effect of different methods of preparing the inoculum*

Large inocula are required for this technique. When the organism is grown on a medium containing excessive amounts of vitamin B<sub>12</sub> (> 0.05 µg./ml.) growth is prolific, but the inoculum thus prepared is useless for assay purposes, as growth occurs even in the absence of vitamin B<sub>12</sub>, presumably because it is stored in the organisms. This difficulty may be overcome as follows. The organism is grown for 16 hr. in a vitamin B<sub>12</sub>-rich medium (10 ml. medium without agar or ascorbic acid but with > 0.05 µg. vitamin B<sub>12</sub>/ml.). The dense overnight growth is washed with saline and resuspended in saline, and 0.2–0.5 ml. of this suspension is used to inoculate 10 ml. of a similar liquid medium free from vitamin B<sub>12</sub>. On further incubation very great growth occurs; after 5–8 hr. there is a dense suspension, which may be used for assay purposes. Probably the vitamin B<sub>12</sub> stored in the parent organisms is used up or distributed amongst daughter cells during this phase of very rapid growth on the deficient medium, thus preventing further growth on the plates in the absence of vitamin B<sub>12</sub>. A technique of this type may also be used in the preparation of inocula of *L. casei* and *L. fermentum* P36 for use in plate assays.

The observation that *L. lactis* does not require vitamin B<sub>12</sub> in the presence of large amounts of ascorbic acid or thiolactic acid suggested that suitable inocula might be grown in the absence of vitamin B<sub>12</sub> but in the presence of these reducing agents. However, such inocula grow prolifically on the plates even in absence of vitamin B<sub>12</sub>. This phenomenon is being investigated; it appears to be due to the synthesis, under anaerobic conditions, of a substance capable of replacing vitamin B<sub>12</sub>.

After consideration of the different methods of preparing the inoculum, the one described first in this section was adopted because it was most suitable for laboratory routine. The concentration of vitamin B<sub>12</sub> used must fall within the limits given; insufficient growth is obtained with less; with more, growth occurs on the test plates even in its absence.

#### *Interference*

As stated by Cuthbertson, Lloyd & Pegler (1951), the response of *L. lactis* to vitamin B<sub>12</sub> is affected by inhibitors of bacterial growth and by deoxyribosides; the former prevent growth and the latter at concentrations of 5 µg./ml. or more cause the zone of exhibition produced with vitamin B<sub>12</sub> to become diffuse, thus interfering with the assay.

*The conditions essential for well-defined response of Lactobacillus lactis to vitamin B<sub>12</sub>*

(1) The medium must be aerobic; (2) reducing agents must be present; (3) excess of oxygen must be avoided (excess oxygen may exert its effect by removing the reducing agent); (4) the dilutions put in the cups must not contain more than 5  $\mu$ g. deoxyribosides/ml.; (5) antibiotics must be absent.

From our results it appears that under highly reducing conditions (or in anaerobic media) vitamin B<sub>12</sub> is not required for growth. These conclusions are also consistent with the observations of Greene, Brook & McCormack (1949) and Koditschek, Hendlin & Woodruff (1949). Further work should show whether this is because under such conditions the vitamin B<sub>12</sub> is synthesized, replaced by another substance that can only be formed anaerobically or concerned only in reactions, such as those involving oxygen or H<sub>2</sub>O<sub>2</sub>, that are not involved in these circumstances.

In the presence of oxygen vitamin B<sub>12</sub> is required, but alone it is insufficient, for a growth factor present in some tryptic casein digests or in tryptic milk digests is also needed. This growth factor is readily destroyed on exposure to oxygen, but is restored to activity or preserved from destruction by 0.1 % thiolacetic acid or 0.1 % ascorbic acid.

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