NOTES ON MICROBIOLOGICAL ASSAYS USING *LACTOBACILLUS LACTIS* DORNER

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The announcement by Shorb[1,2,3] that *Lactobacillus lactis* Dorner responded to a factor present in liver extracts to an extent roughly proportional to their clinical value, opened up the possibility of a microbiological assay of anti-pernicious anæmia preparations. The isolation of the growth factor by Rickes *et al*[4], who named it vitamin B_{12}, and the demonstration by West[5] of the anti-pernicious anæmia potency of Vitamin B_{12}, strengthens this possibility.

Most types of bacteria moulds, etc., are able to synthesise vitamin B_{12} but several organisms have been found which lack this property and these have been tried for assay purposes. The present communication deals with a method of assay in liquid culture using *L. lactis* Dorner.

Shorb[2,3,6] and Shive[7] have drawn attention to a marked tendency for this organism to behave in an inconstant manner under certain conditions. This uncertainty has been confirmed by the unpublished experiences of many workers in this field and, for many months, investigations in these laboratories were hampered by erratic assay results. The technique finally evolved and described below seems to be free from the disabilities reported by other workers.

The principles utilised in the final method are identical with those influencing all other microbiological assays (Shaw[8]). The test inoculum must be minimal. The organisms in the test inoculum must be young, in a highly active phase of growth, and to have been maintained on a complete medium. The medium must be free from other growth factors. The difference between *L. lactis* Dorner and other organisms is that the former is much more prone to variation formation. It was found that when optimum conditions had been established, the assays were more consistent than with any other microbiological assay performed in these laboratories.

The basal medium employed consists of sulphuric acid-hydrolysed casein with added tryptophane, the usual members of the vitamin B complex group and tween 80. T.J. factor, another essential supplement, is supplied as a concentrate of potato extract consisting of the ether precipitate from a phenol extract of the fraction insoluble in alcohol (80 per cent.) but soluble in alcohol (50 per cent.). One advantage of this source of T.J. factor is that unlike with enzyme digested casein used by Shive,[7] or with tomato juice, used by Shorb,[1,2,3,6] it is possible to use a large excess of T.J. factor without at the same time adding *L. lactis* Dorner factor. Another advantage is that since the current batch of concentrate is used at a final dilution of 1/50,000, it is possible to use the same batch of potato extract over a considerable period. All batches of tween 80 are not equally suitable. One sample which did not give a clear 10 per cent. solution in water was useless.

The reaction of the final medium is adjusted to *p*H 5·5 and the glucose, potato extract and preparation to be tested are added aseptically to the previously autoclaved basal medium. If the glucose is added to the medium prior to autoclaving, the resultant broth may support the growth of *L. lactis* Dorner in the absence of liver extract or other vitamin B_{12} containing material. Under these circumstances the " blank " is high and may even approach maximum turbidity. Autoclave treatment at *p*H 7 to 8 is more likely to upset the assay than when the *p*H is approaching 5·5 at which *p*H the phenomenon is rarely observed. One does not always obtain this growth potentiation on autoclaving and one possible explanation of failure to do so may be that some samples of glucose solution may become acid on autoclaving. If the *p*H is lowered before the reaction between glucose and media constituents can occur, it would be equivalent to autoclaving at a low safe *p*H.

The strain used was isolated as a single colony from soya bean medium and has been maintained for upwards of 150 daily sub-cultures on peptone agar, fortified with proteolysed whole liver, tween 80 and potato extract. Its behaviour has remained constant throughout this series of cultivation.

For assay purposes an overnight agar slope culture is washed into 10 ml. of basal medium fortified with proteolysed whole liver, and this allowed to incubate for 5 to 6 hours at a temperature of 37°C. The broth culture is centrifuged, the sedimented organism washed 2 or 3 times with basal medium deficient in vitamin B_{12} and finally suspended to an opacity of approximately 1/5th, in either vitamin free basal medium or saline, immediately before use. Three drops of this suspension are used to inoculate 10 ml. of test medium.

Growth is usually satisfactory after 16 to 18 hours incubation, but, if the inoculum is too small or the organism is not in good condition, incubation up to 48 hours may be necessary. Exposure of the washed organism to saline solution for more than about $1\frac{1}{2}$ hours may convert the organism from a plump bacillus, staining solidly Gram-positive, into a thin one with the body of the organism Gram-negative showing Gram-positive beads simulating the appearance of a chain of streptococci. Further exposure to saline solution might even result in a completely Gram-negative filamentous organism from which it is difficult to get a satisfactory culture. In contrast to the report of Shorb[3] we have not found that the length of incubation affects the assay values. The general levels of growth are slightly higher but the test solution is affected equally with the control. Probably as a consequence of the aseptic addition of glucose, the test medium is water white, and during the period when glucose was autoclaved with the medium it was found that, in general, a low blank was obtained when the colour of the medium was minimal, the colour being presumably an index of glucose decomposition.

To ensure regular results, it is essential to use constant-bore test tubes in order to have a constant surface-area/volume ratio. Incubation is best performed in a large room-incubator and the test tubes must not be shaken during the growth period. Sterility of the glucose is ensured by

the candle filtration of a 40 per cent. solution. Liver extracts in ampoules are assumed to be sterile, otherwise dilutions are made from preparations which have been allowed to stand in the presence of 1 per cent. phenol. The final dilution of the liver extract in the final medium is usually so great as to eliminate the effect of any phenol carried over. Where necessary, the preparation to be assayed is autoclaved and this does not appear to have any deleterious action. It is however best to avoid autoclaving the test sample.

It has been found convenient to make the basal medium up to 9/10ths final strength, to distribute this into aluminium capped test tubes in 9 ml. quantities and then to autoclave these tubes in lots of about 500 tubes. The tubes are prepared for use by adding the requisite amounts of glucose, potato extract and vitamin B_{12} preparation all in a volume of 1 ml. The autoclaved tubes may be used at any time up to 14 days after autoclaving without affecting the assay in any way; thus enabling a large number of assays to be conducted with medium is known to be identical in every respect.

The optimum conditions for autoclaving are obtained by the use of a large autoclave in which the tubes are placed in special racks so arranged that there is 1 in. free space around each tube. The autoclave is fitted with a recording thermometer and so it is possible to heat up during 10 minutes, to maintain at a temperature of 115°C. for 10 minutes and to take the same period to cool down. There is also available a permanent record of the autoclave history of that batch of tubes.

According to Shorb[6], *L. lactis* Dorner maintained on yeast-extract-agar with or without tomato juice, gives irregular results, and growth may be inhibited by some liver extracts. The strain used in these laboratories with the described technique gives very reproducible results and the dose-response curve for all liver extracts assayed, for pure vitamin B_{12} and thymidine, have been found to be identical whether assayed individually or in mixture. Thymidine has only 1/10,000th of the activity of vitamin B_{12}. Values obtained from the assay of mixtures are invariably the sum of two components. Inhibitors might be expected to affect the shape of the growth response curve and the values obtained on blending.

Growth-response curves are established for each assay by using 5 levels of the standard preparation. It is desirable to put up an equal number of levels of unknown, endeavouring to arrange the dilutions so that the reading of each level of the test coincide with the corresponding levels in the control series. For accurate work it is desirable to use 6 tubes at each level and to repeat the assay at least 3 times. Ordinarily 3 tubes at each of 3 or 4 levels provide a fairly accurate estimation. A typical growth response curve is shown in Figure 1.

Lester Smith and Cuthbertson[9] reported the presence of four substances with growth activity for *L. lactis* Dorner which may be present in liver extracts. These substances have different migration velocities on paper strip chromatograms when developed with aqueous butyl alcohol. Two of the substances, both with anti-pernicious anæmia activity, migrate less rapidly than riboflavine, and two, with no anti-pernicious anæmia

potency, migrate more rapidly than riboflavine, one being thymidine, which is found very near to the advance front. The possibility of other *L. lactis* Dorner-active materials being present in a liver extract in addition to vitamin B_{12} must therefore be taken into consideration. This possibility has been dealt with by subjecting various extracts to paper strip chromatograph (Shaw[10]). A spot of extract or other preparation (1/200th ml. of an extract assaying at 10 μg/ml. is satisfactory) is placed on a 1 cm. strip of Whatman No. 1 filter paper and, after drying, this strip is suspended in normal butyl alcohol, saturated with water, so that the butyl alcohol level is about 2 cm. below the applied spot. The butyl

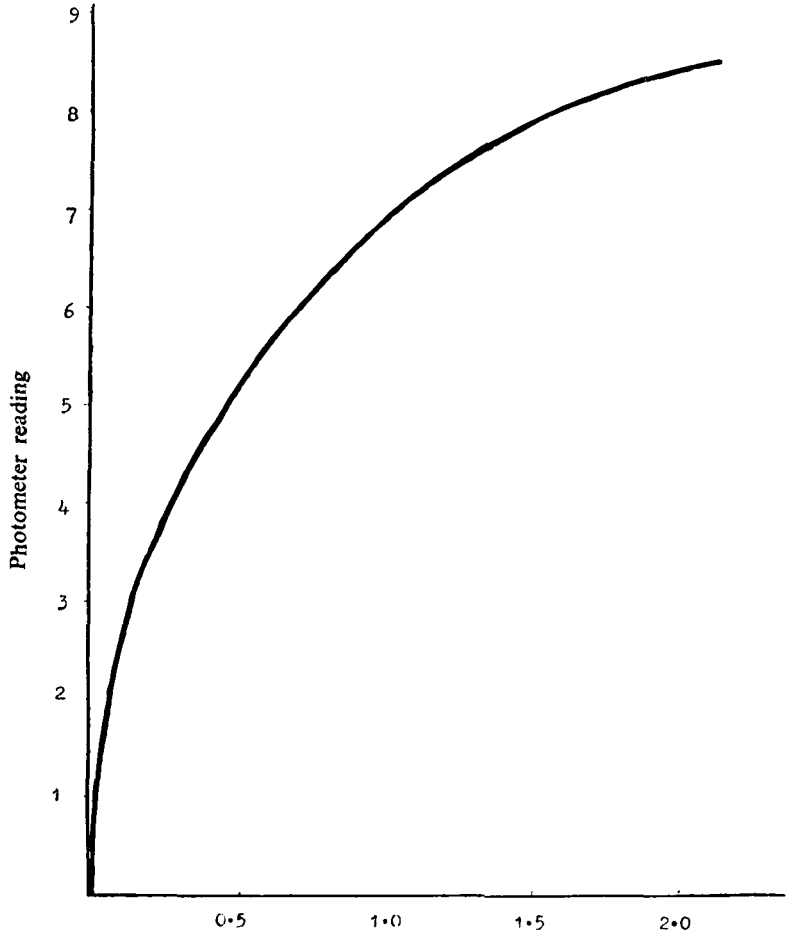

FIG. 1.—Growth-response curve vitamin B_{12}, *Lactobacillus lactis* Dorner. Average of 25 tests. 10 ml. tubes—1 cm. cell.

alcohol is contained in the bottom of a glass cylinder and the sides of the cylinder extend to about 13 cm. above the spot. The whole is protected

from light and the butyl alcohol travels up the strip, evaporates at the top of the cylinder and deposits all of the rapidly migrating material at this point of the strip at the advanced front.

A small amount of riboflavine may be placed on the same spot as the liver extract and after about 48 hours development, it will be found that the advance front is 13 cm. from the spot and a riboflavine belt (identified by examination under ultra violet light) is found about 6 cm. from the spot. The strip is cut at this point, each portion extracted with water and the extract assayed, thus giving the amount of activity which migrates both less and more rapidly than riboflavine.

It was found that the speed of migration of the main *L. lactis* Dorner-active fraction was so much slower than that of the other fractions that latterly the strip has been allowed to develop for a longer period, i.e., 14 to 21 days, when it has been found that most of the activity has left the spot, accumulating in a peak about $2\frac{1}{2}$ cm. from the spot. There is then a considerable length of filter paper which is devoid of activity and the rapidly migrating growth materials accumulate at the advance front. The strip is examined by cutting it up into 0·5 cm. sections, extracting each with water and assaying separately. A typical liver extract gives results as shown in the histogram in Figure 2.

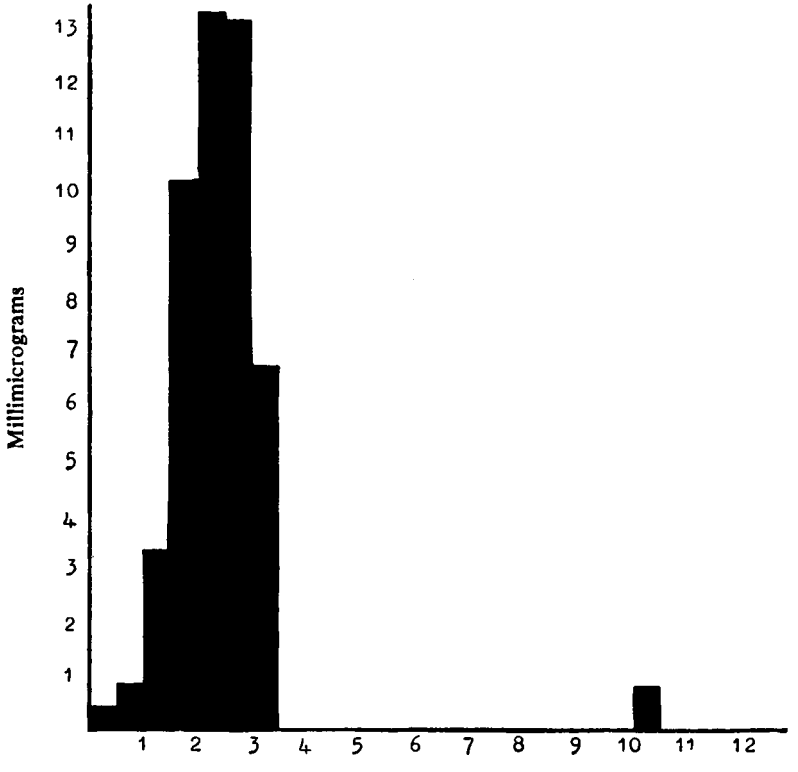

FIG. 2.—Paper chromatograph of vitamin B_{12} in liver extract Product "A," 0·005 ml. developed for 18 days.

SUMMARY

A method has been described for conducting microbiological assay of material containing vitamin B_{12}. Over a long period regularly reproducible results have been obtained. Means have been described briefly for assessing the extent of interference with the assay of inhibiting materials and growth factors for *L. lactis* Dorner other than vitamin B_{12}.

APPENDIX

1. *HEPAMINO-PEPTONE-TWEEN AGAR.* (For strain maintenance.)

Evans Bacteriological Peptone	10 g.
Hepamino (Evans Medical Supplies)	1 g.
Tween 80	1 ml.
Glucose	50 g.
Potato Extract double the concentration used in final assay medium.	
Tap Water	to 1000 ml.

Adjust *p*H to 6·8, heat to 70°C. for 10 minutes, filter, add 10 g. of agar. Filter, tube in 10 ml. quantities. Autoclave for 10 minutes at 10 lb. steam pressure.

2. *ACID HYDROLYSED CASEIN.* (Modified from Mueller *et al*[11].)

Technical Casein	1000 g.
Concentrated Sulphuric Acid	400 ml.
Distilled Water	2600 ml.

Reflux for 50 hours, adjust *p*H with calcium hydroxide to *p*H 7·6, filter and wash precipitate with hot distilled water. Decolourise with activated charcoal. Dilute decolourised solution to the desired nitrogen content.

3. *ASSAY MEDIUM* (Double strength).

Acid Hydrolysed Casein to provide in double strength medium	0·32 per cent. of nitrogen
Tryptophane Solution (0·1 per cent.)	200 ml.
Cystine Solution (0·4 per cent.)	50 ml.
Salt Solution "C"	40 ml.
Dipotassium hydrogen phosphate	5 g.
Potassium dihydrogen phosphate	5 g.
Distilled Water	to 900 ml.

Adjust *p*H to 6·9, heat to 70°C. for 10 minutes, filter, add 2 ml. of Tween 80, uracil, adenine, guanine and all vitamins as for *Lactobacillus Helveticus* (Shaw[8]). Dilute with distilled water to 1000 ml.

Note 1.—Potato extract is added to a concentration of double that which will give maximum growth with 2 μmg. of Vitamin B_{12}/10 ml. in 16 to 18 hours.

Note 2.—Most batches of glucose B.P. will substitute for glucose of A.R. quality.

Note 3.—A solid liver preparation standardised by Dr. Rickes of Messrs. Merck and Co., at 0·4 μg/mg. has been used as reference standard.

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