The Assay of Vitamin B₁₂. Relationship between Growth Response of *Lactobacillus leichmanii* 313 in Tubes and Diffusion of Oxygen into the Medium

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The microbiological tube assay for vitamin B₁₂ with micro-aerophilic *Lactobacilli* as test organisms requires careful control of E_h in the assay medium if uniform responses to the vitamin are to be obtained. The presence of reducing substances and degree of aeration of the test medium are the main factors in determining this E_h . Apart from substances that may be added in the dilutions of the test sample, the main source of reducing material is the interaction of constituents of the medium during sterilization in the autoclave. A rigorously standardized autoclaving procedure, as described by Shaw (1948), is the best method of controlling this variable. The effects of aeration and reducing substances on the growth response of *Lactobacillus lactis* Dorner to vitamin B₁₂ have been described by Kocher (1949) and by Koditschek, Hendlin & Woodruff (1949).

When attempting to adapt our previously described tube assay method (Lees, Emery & Tootill, 1951) to an agar plate technique for the examination of paper strip chromatograms, we found incorporation of a reducing agent in the medium necessary for growth response to vitamin B₁₂ in the relatively thin agar layer. Because the reducing agent is indispensable in the plate assay and because of an observation relating the depression of response in the turbidimetric tube assay with abnormally wide tubes, we have examined in more detail the effects of the diffusion of oxygen into the medium on the growth response of *Lb. leichmannii* 313 to vitamin B₁₂ and have tried to assess their magnitude.

EXPERIMENTAL

The conditions of assay employed were those described by Lees *et al.* (1951). Briefly, a sterile medium adequate for the nutritional requirements of the test organism, but free of vitamin B₁₂, is inoculated with a washed suspension of the test organism; after incubation graded responses are obtained according to the amounts of vitamin B₁₂ added to the tubes. The response is determined by reading the turbidity directly in the Spekker photoelectric absorptiometer on a logarithmic scale, which gives a reading approximately proportional to the amount of growth. To obtain the most precise measure of the magnitude of the effects, these conditions were varied so as to involve bulk inoculation of the test organism and vitamin B₁₂, whenever the design of the experiment permitted it.

In view of the importance of autoclave timing when the

basal tube medium is being sterilized, we give below the essential details of our procedure. The metal racks containing spaced tubes were placed in the autoclave and subjected to free steaming (no rise from atmospheric pressure) for 5 min. The valves were closed and the pressure was allowed to reach 15 lb./sq.in. during an interval of 1.5 min. The steam was then turned off and the pressure allowed to equilibrate with the atmosphere in 1.5 min. by adjustment of the exhaust valves. The racks were immediately removed and cooled in water.

RESULTS

To verify our observations relating the depression of growth response of the *Lactobacillus* in certain tubes to their abnormal width, we first examined the response of the test organism to vitamin B₁₂ in a series of stock tubes of increasing internal diameter, each tube containing a constant volume of medium.

It was found that with doses of vitamin B₁₂ below 0.23 mμg./tube, some tubes showed no growth whatsoever, whereas with doses in excess of 1.20 mμg./tube, many tubes showed no gradation in turbidity against further increase of dose since maximum growth had already been obtained.

Accordingly, the responses for doses in the range 0.23–1.20 mμg./tube were analysed and are shown in Table 1 with the accompanying analysis.

The analysis shows that a highly significant linear component of regression of growth on surface area exists, with a smaller, but still highly significant, deviation from linear regression.

It was considered that this deviation from the linear component was most probably due to the effect of depth of medium on the growth response, although the deviation could also be explained by the existence of a curved regression of response on surface area. An experiment with uniform tubes, each containing the same volume of medium but having varying surface areas exposed to the atmosphere, served to show that a linear regression between growth and surface area was sufficient to account for the data when depth was maintained constant. To obtain the varying surface areas a series of paraffin wax annuli of known internal diameters were floated on the surfaces of the medium in the tubes, the periphery of each annulus being sealed to the inner wall of the assay tube by means of a small flame.

Table 1. *Growth response related to area of tube exposed to air*
(Growth response is given as turbidity readings using the Spekker absorptiometer.)

Tube internal cross-sectional area (sq.cm.)	Doses of vitamin B ₁₂ (μg./tube)				
	0.23	0.35	0.53	0.80	1.20
	Growth response				
1.54	0.78	1.13	1.28	1.40	1.56
	0.80	1.11	1.19	1.42	1.56
2.14	0.62	0.98	1.10	1.37	1.48
	0.74	1.03	1.12	1.36	1.45
4.30	0.06	0.11	0.24	0.46	0.56
	0.06	0.07	0.22	0.40	0.77

Analysis of variance					
Source of variation	Degrees of freedom	Squares	Mean square	Ratio	Probability
Regression on tube surface area	1	5.134,12	5.134,12	1989.97	$P < 0.001$
Deviation from area regression	1	0.064,84	0.064,84	25.13	$P < 0.001$
Differential response of doses on tubes	8	0.086,04	0.010,76	4.17	$P < 0.010$
Doses	4	1.892,42	0.473,11	—	—
Error	15	0.038,65	0.002,58	—	—
Total	29	7.216,07	—	—	—

Table 2. *The effect of paraffin wax annuli on growth response*

Diameter of hole (mm.)	16	11	9	8	7	6	5	4	0
Duplicate responses	0.70 0.64	0.84 0.95	0.78 1.03	0.93 0.92	0.90 1.03	0.97 0.91	0.99 1.05	0.86 1.00	0.98 1.09
Mean response	0.670	0.895	0.905	0.925	0.965	0.940	1.020	0.930	1.035

Analysis of variance					
Source	Degrees of freedom	Squares	Mean square	Ratio	Probability
Regression on area of medium exposed	1	0.1612	0.1612	21.65	$P \approx 0.001$
Deviation	7	0.0171	0.00244	0.33	Not significant
Error	9	0.0670	0.00745	—	—
Total	17	0.2453	—	—	—

Table 2 presents the results and analysis of this experiment, showing a linear regression of growth response on the area of the medium exposed to the air, with a deviation from this regression well below significance.

These results made us reasonably confident that the effects were due to atmospheric oxygen and, since the assay medium is stationary during growth of the test organism, it was assumed that oxygen entered the medium by solution at the surface and reached the lower level by diffusion only. It was further assumed that inhibition of growth due to dissolved oxygen would, with increasing depth, decrease asymptotically to a finite value or zero, and that the growth in any given lamina of medium would thus be related to its depth below the surface of the medium. The turbidity of an infinitesimal lamina being impossible to measure, the contents of the tubes were dispersed by shaking before turbidity determinations, to give a measure

of the average turbidity throughout the medium. It was thought that the limiting value of inhibition would be attained at not too great a depth below the surface and that the average turbidity we measured could therefore be considered as a dilution of the maximum growth attainable below this depth with the subnormal growth obtained at lesser depths. Thus it was expected that the average growth throughout the medium would be inversely related to the depth, at least beyond the point at which the limiting value was obtained. Accordingly an experiment was set up with varying depths of medium in tubes selected for uniform internal diameter. The statistical analysis of the results in Table 3 shows that the inverse relationship was applicable to our experiment in depths of medium corresponding to 8 ml. and upwards in 6 × 1 in. assay tubes.

The assumption was made that the inhibition, I , of growth in an infinitesimal lamina at a depth

Table 3. *Growth response of test organism according to the depth of the assay medium*

Volume in 1 in. tubes (ml.) ...	2	4	6	8	10	12	14	16	18	20
Replicate responses	0.50 0.50 0.52	0.58 0.59 0.58	0.66 0.70 0.70	0.79 0.83 0.80	0.91 0.91 0.93	1.01 1.01 1.01	1.07 1.07 1.10	1.14 1.15 1.10	1.12 1.19 1.15	1.16 1.15 1.17
Mean responses	0.507	0.583	0.687	0.807	0.917	1.010	1.080	1.130	1.153	1.160

Analysis for reciprocal relation between mean response and depth of medium from 8 ml. upwards

Source	Degrees of freedom	Squares	Mean square	Ratio	Probability
Regression	1	0.31996	0.31996	772.85	$P < 0.001$
Deviation	5	0.00231	0.000462	1.11	Not significant
Error	14	0.00580	0.000414	—	—
Total	20	0.32807	—	—	—

corresponding to a volume v in the tube would be given by the formula

$$I = A \exp(-\frac{1}{2}k^2v^2).$$

It was observed that this formula is similar to one given by Cooper & Woodman (1946) for the diffusion of antiseptics through agar gels, where volume has replaced distance as a variable, since in tubes of constant diameter volume is proportional to depth. Further, since a constant time of incubation was employed for all experiments, time, which appears as a variable in Cooper & Woodman's formula, has been included in the constant k . In effect, the equation assumes that the inhibition is simply related to the oxygen concentration obtained by diffusion.

If g represents growth in a lamina of medium corresponding to volume v

g_0 growth at the surface of the medium, i.e., in conditions of oxygen saturation

and g_∞ growth at 'infinite' depth, i.e., in complete absence of diffused oxygen, then

$$g = g_\infty - I,$$

$$g = g_\infty - A \exp(-\frac{1}{2}k^2v^2),$$

$$\text{and} \quad \frac{g_\infty - g}{g_\infty - g_0} = \exp(-\frac{1}{2}k^2v^2).$$

The measurements are made on the average growth obtained throughout the tube, as described above; they can be obtained from the formula by summing the growth in all laminae and dividing the result by the volume concerned. Thus if $\bar{g}$ represents the average growth obtained in a volume v , then

$$\frac{g_\infty - \bar{g}}{g_\infty - g_0} = \frac{1}{kv} \exp(-\frac{1}{2}k^2v^2) \, d(kv).$$

Define $\phi(X)$ by

$$\phi(X) = \int_0^x e^{-t^2} dt.$$

$$\text{Then} \quad \frac{g_\infty - \bar{g}}{g_\infty - g_0} = \frac{1}{kv} \phi(kv),$$

and thus $\bar{g}$ is linearly related to $\frac{1}{v} \phi(kv)$, whose value for any specified volume is known when k is known. The value of k may be chosen so as to make the regression of $\bar{g}$ on $\frac{1}{v} \phi(kv)$ as nearly linear as possible.

(Values of ϕ for any value of the product kv may be read very easily from tables of the normal probability integral.)

An approximate value for k was determined graphically and the values of $\frac{1}{v} \phi(kv)$ for the volumes used in the experiment were calculated for graded values of k in the neighbourhood of the approximate value previously obtained. The truth of the above hypothesis relating aeration of test medium to inhibition of the test organism implies linear regression between the experimentally observed results and the values of $\frac{1}{v} \phi(kv)$ for the maximum likelihood value of k .

Thus the sum of squares attributable to this linear regression was calculated for the graded values of k and the maximum value determined by interpolation; this maximum value corresponds to a minimum mean square value for the deviation from the regression, so determining the maximum likelihood value of k .

The tabulated values of the above functions and the corresponding derived results (based upon the maximum likelihood value of $k = 0.222,54$) are given in Table 4.

The analysis in Table 5 shows this deviation to be non-significant, and the formula therefore accounts for all the relevant variation experienced and is shown to be statistically valid.

The value of k determined in the above manner enabled the constants of the linear regression between the experimental results and $\frac{1}{v} \phi v$ to be evaluated, thus giving values for g_0 and g_∞ . The values of g and $\bar{g}$ are shown graphically in Fig. 1, the experimental results being superimposed as circles.

Table 4. *Experimental results shown with maximum likelihood values of theoretical formula*

v = volume in tubes	$\phi'kv = \exp(-\frac{1}{2}k^2v^2)$	g = theoretical growth in lamina of medium corresponding to volume v	$\frac{1}{kv} \phi(kv) = \frac{1}{kv} \int_0^{kv} \exp(-\frac{1}{2}k^2v^2) d(kv)$	$\bar{g}$ = calculated responses	Mean experimental responses
0	1.000,00	0.467	1.000,00	0.467	—
2	0.905,74	0.561	0.967,95	0.499	0.507
4	0.672,88	0.794	0.882,26	0.584	0.583
6	0.410,07	1.056	0.768,00	0.699	0.687
8	0.204,99	1.261	0.561,16	0.815	0.807
10	0.084,06	1.382	0.548,52	0.918	0.917
12	0.028,28	1.438	0.465,77	1.001	1.010
14	0.007,80	1.458	0.401,54	1.065	1.080
16	0.001,77	1.464	0.351,86	1.114	1.130
18	0.000,33	1.466	0.312,86	1.153	1.153
20	0.000,05	1.466	0.281,59	1.185	1.160

Table 5. *Analysis to test the statistical validity of theoretically derived formula*

Source	Degrees of freedom	Squares	Mean square	Ratio	Probability
Function	2	1.611,310	0.805,655	2237.93	$P < 0.001$
Deviation	7	0.004,357	0.000,622	1.73	Not significant
Error	20	0.007,200	0.000,360	—	—
Total	29	1.622,867	—	—	—

Further experiments were conducted, designed to examine the effect of various levels of vitamin B₁₂ on the shape of the inhibition curve shown by the lower line in Fig. 1. The relationship between the

Fig. 1. Experimental turbidities (○) obtained at varying depths superimposed upon the theoretically derived relationship between depth of medium and turbidity of growth: Curve I, $\bar{g} = A - \frac{B}{kv} \int_0^{kv} \exp(-\frac{1}{2}k^2v^2) d(kv)$.

Assumed curve showing the turbidity in individual laminae of medium at varying depths: Curve II, $g = A - B \exp(-\frac{1}{2}k^2v^2)$.

mean growth response and the volume of medium in the tube for three levels of vitamin B₁₂ is shown in Fig. 2. The experiment could have been improved by using a logarithmically spaced series of doses.

DISCUSSION

The curves in Fig. 2 show the relationship between volume of medium and density of growth obtained at the end of incubation. A simple interpretation of this gradation of growth in the light of oxygen

Fig. 2. Relationship between growth of *Lactobacillus leichmannii* 313 at varying depths for three doses of vitamin B₁₂.

diffusion is not possible, because growth takes place over a period of hours and oxygen concentration at any given depth varies with time during this period. The growth of the organism in any horizontal

lamina is taking place in the presence of oxygen concentration increasing with time, but the curves of oxygen concentration with respect to depth at all times are similar in shape, thus supporting an interpretation of the terminal incubation conditions relating inhibition of growth to oxygen diffusion from the atmosphere. The apparent inhibition of the test organism noted may be accentuated by a gradual settling of the organisms during incubation.

To minimize the interference from oxygen in the test medium in routine tube assay of vitamin B₁₂, it is necessary to use tubes carefully selected for uniform internal diameter and to ensure that the conditions of aeration of the medium, after autoclaving, are as nearly as possible identical for the standard test sample assay tubes. This can be conveniently achieved by allowing the autoclaved tubes to attain atmospheric equilibrium before inoculation or by allowing only an insignificant interval of time to elapse between inoculation of standard and test sample assay tubes, a procedure only possible when few unknowns are being assayed against one standard preparation.

The accuracy of any microbiological assay depends to a certain extent upon the slope of the dose-response curve. Examination of Fig. 2 shows that this slope, i.e. the difference in absorptiometer reading for a standard dose increment, increases rapidly until a volume of 15–20 ml./tube is used, when no further practical advantage is gained by the use of larger volumes. With this information, we improved our assay method by using narrower tubes with our original volume of 10 ml., thus increasing the depth of the medium, which achieves the same end as an increase in its volume. We recommend that workers performing tube assays employing micro-aerophilic test organisms should examine the slope of the dose response curve with respect to the depth of medium in the assay tubes, obtaining such a relationship as is shown in Fig. 2, whence the most suitable depth of medium can easily be determined.

In determining the most suitable volume of medium for microbiological tube assays employing micro-aerophilic test organisms, it should be borne in mind that this may be affected by constituents of the medium. Schweigert, Guthneck & Schneid (1950), employing medium volumes of 2 ml./tube, found that the replacement of cystine with cysteine greatly improved the growth of *Lb. leichmannii* 313; it is clear that in this small volume the reducing properties of cysteine are effective in overcoming the inhibition due to the highly aerobic medium. This interpretation is supported by the fact that for optimum growth of *Leuconostoc mesenteroides* much lower levels of cysteine and cystine are required. Cystine, when autoclaved with hydrolysed casein, is partially converted to a substance with growth-promoting properties much exceeding those of the original cystine added to the medium. Cysteine added aseptically to the medium after autoclaving also yields good growth, suggesting that cystine is, to a certain extent, converted to cysteine, whose reducing properties are the important factor in promoting good growth of lactobacilli.

SUMMARY

1. The relationship between the growth response of *Lactobacillus leichmannii* 313 to vitamin B₁₂ and the oxygen content of the assay medium has been investigated.

2. A simple theory of inhibition of the test organism on the basis of diffusion of atmospheric oxygen has been postulated and found to account satisfactorily for the experimental observations.

3. It is recommended that the optimum conditions for microbiological assays with micro-aerophilic test organisms should be established after examining the growth response of the organism along lines similar to those described in the paper.

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