A note on the comparison of the microbiological potencies of two different growth factors with special reference to the assay of desdimethylcyanocobalamin

FORD *et al.*[1] and ourselves[2] have isolated a vitamin B_{12} analogue that differs from cyanocobalamin in lacking the two methyl groups of the 5:6-dimethylbenziminazole portion. (For purposes of this communication only the analogue will be referred to as desdimethylcyanocobalamin (DDMC).) FORD *et al.*, using an *Escherichia coli* tube assay, found DDMC to have only 27% of the activity of cyanocobalamin, whereas we found it to be 2.4 times as active in tests against the same organism in a plate assay. The work described here was undertaken to study this discrepancy.

We thought that the different relative potencies of the two compounds in the two types of assays had some connection with the main difference between the two assay procedures themselves: in a plate assay the test substance diffuses through a solid medium containing the test organism, and the size of the zone of growth caused by the substance will depend to a certain extent both on its simple rate of physical diffusion and on the avidity with which it is taken up by the test organism. When assaying an unknown amount of a substance against standard amounts of the same substance these factors will influence all samples in the same way and will therefore cancel out, but they may not be irrelevant when two different substances are compared in a plate assay.

In the first series of experiments *E. coli* (mutant) assay plates (on BURKHOLDER's[3] medium) and the two vitamin solutions were kept at 37° before the solutions (3 drops containing 0.4, 0.2, 0.1 μg/ml of cyanocobalamin and DDMC) were placed in the assay cups in a random design. The pre-equilibration at 37° was carried out so that the organism could begin growing as soon as the vitamin solutions were added, thus avoiding as far as possible diffusion of the substances in the absence of growth. After incubation at 37° overnight the zone diameters were measured and plotted in the normal fashion against the logarithms of the vitamin concentrations. Straight lines were obtained for both substances, but the slopes were different. When the DDMC zones were read on the cyanocobalamin graph, DDMC gave much higher values, the difference increasing with concentration; the factors by which the "titres" differed from that of cyanocobalamin are shown in Table I.

TABLE I

PLATE ASSAY COMPARISON OF CYANOCOBALAMIN AND DDMC

Cyanocobalamin or DDMC (μg/ml)	*G/A*				
					Average
0.4	2.53	3.40	2.98	2.73	2.91
0.2	2.30	2.60	2.10	2.38	2.34
0.1	2.00	1.97	1.76	1.97	1.92

G = Concentration of DDMC obtained from cyanocobalamin graph.
A = Actual concentration of DDMC.
Each figure represents the average of six determinations.
Each vertical column represents values obtained from one plate.

In the second series of experiments the two solutions were applied to uninoculated plates and allowed to diffuse for several hours at 37°. A layer of inoculated agar medium was then poured over the plates and incubation at 37° was continued. This technique was intended to minimise differences due to differential uptake of either substance by the test organism. Since there was no bacterial uptake during the first hours, the substances diffused much further, causing larger though more diffuse zones of growth. Graphs were again constructed and the G/A ratios were calculated. They were between 1.2 and 1.4, *i.e.* appreciably lower than in the first series.

From the results of these experiments it appeared that the main cause for the apparent higher relative activity of DDMC lay in preferential uptake of cyanocobalamin by the test organism.

This was put to the test in a more direct way by studying the uptake of the ^{60}Co-labelled substances by washed *E. coli* suspensions. *E. coli* cells grown on peptone-water for 18 hours were spun down, washed three times in saline and finally re-suspended in saline at approximately $3 \cdot 10^9$ organisms/ml. To 10 ml aliquots 2 ml portions of the labelled substances were added in such a way that the amount of labelled material was the same in all tubes, but the total concentrations of the respective substances were different. The tubes were allowed to stand with occasional

shaking for 1 hour at room temperature (results after 18 hours standing were similar), the contents were spun and the cells washed with saline and spun again. Aliquots of the re-suspended cells and the combined supernatants and washings were "counted" in a liquid counter. The results showed that cyanocobalamin was taken up by the cells more readily than DDMC. This was shown more clearly in another suspension when mixtures of cyanocobalamin and a small amount of labelled DDMC (and DDMC with a small amount of labelled cyanocobalamin) were used. The results of these experiments were summarised in Table II.

TABLE II

THE UPTAKE OF LABELLED CYANOCOBALAMIN AND DDMC BY WASHED *E. coli* CELLS

Substance	*μg/12 ml suspension*		*% of counts*	
			supernatant	*cells*
DDMC	49.1		96	1
	5.0		100	3
	0.5		92	11
	0.05		32	67
Cyanocobalamin	48.8		110	1
	4.9		104	2
	0.5		87	14
	0.05		23	74
DDMC* + cyanocobalamin	0.05	4.9	99	2
	0.05	0.82	94	3
	0.05	0.40	88	13
	0.05	0	41	55
Cyanocobalamin* + DDMC	0.05	4.9	90	8
	0.05	0.82	70	35
	0.05	0.40	38	60
	0.05	0	23	75

* The counts are expressed as % of the total count given by similar amounts of the respective radioactive substances in 12 ml water.

Finally the potencies of the two substances were compared by an *E. coli* tube assay in a medium resembling that of Burkholder, except that it contained no citrate and had a slightly different amino acid composition. Concentrations of the two substances between 0.00002–0.0008 μg/ml were examined. Again the two response curves had different slopes. When the DDMC values were calculated from the cyanocobalamin graph, it was found that at the higher concentration range DDMC appeared invariably more potent than cyanocobalamin, whereas at the lower concentration range the order was reversed. The DDMC/cyanocobalamin potency ratios so obtained lay between 1.45 and 0.5, but large day-to-day variations in these ratios were encountered.

Preferential uptake of cyanocobalamin by the test organism does not, without further assumptions, explain either the anomalies of the tube assay results or the apparent increase with concentration of the DDMC/cyanocobalamin potency ratio by the plate assay. The results however do indicate that it is meaningless to state which of the two is the more potent growth factor, unless the test conditions are rigidly defined. A reason for publishing this note is to show why it is likely that similar difficulties may be encountered whenever the microbiological potencies of two different substances are compared.

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