THE ROUTINE ASSAY OF B₁₂ IN SERUM

BY

A. I. TIFFIN* AND G. M. WILLIAMSON

From the Department of Bacteriology, the School of Medicine, Leeds

(RECEIVED FOR PUBLICATION NOVEMBER 25, 1957)

The occasional request for the estimation of vitamin B₁₂ in serum presents a difficult problem to the routine laboratory. In designing a suitable method to give reliable results when used at infrequent intervals the following modification of a technique described by Spray (1955) has been devised. It was irregularities in the response of the test organism, *Lactobacillus leishmanii*, as determined by replicate assays, which led to the alterations described. These irregularities were traced to slight differences in inoculum size, to errors in the measurement of small volumes of the standard B₁₂ solutions, and to unequal agitation of the culture tubes when incubated in a mechanically stirred water-bath. This last irregularity led to the observation that *Lactobacillus leishmanii* grows better in unaerated or anaerobic conditions. The modified technique described below has, in our hands, given reproducible results in assays carried out at very long intervals, even as long as five months.

Method

Glassware and Metal Caps.—At the outset, new glassware was purchased, and, after cleaning in 10% hot nitric acid followed by thorough rinsing in distilled water, it was allotted to and kept for specific uses. All cleaning equipment, such as test-tube brushes, are likewise kept for one purpose only. Between each assay the same rigid procedure is adhered to. In this way contamination with vitamin B₁₂ is avoided or, at least, kept to a minimum.

The aluminium caps for the test-tubes were first washed by boiling in water to which a detergent had been added and then well rinsed in distilled water. Between each assay they are simply boiled and rinsed in distilled water. Care must be taken in the cleansing of these caps, for during autoclaving some of the medium is deposited on the cap, a further source of error.

Organism.—The organism used is *Lactobacillus leishmanii* (N.C.I.B. 7884).

*Present address: Department of Chemistry, The Royal Holloway College, London.

Medium.—The medium is similar to that recommended by Spray (1955), and for convenience is made up in the following manner.

Solution A

Acid-hydrolysed casein, sufficient to give	...	...	2.7 g. total nitrogen
Sodium acetate, hydrated (anhydrous)	...	...	10 g. (6 g.)
KH ₂ PO ₄	...	...	2.5 g.
K ₂ HPO ₄	...	...	2.5 g.
Tryptophane	...	...	100 mg.
Cysteine HCl	...	...	100 mg.
Adenine	...	...	10 mg.
Guanine	...	...	10 mg.
Uracil	...	...	10 mg.
Xanthine	...	...	10 mg.
Salt solution B	...	...	20 ml.
Distilled water to	...	...	400 ml.

The acid-hydrolysed casein and salts are mixed with part of the water, heated to 70° C., and filtered. The tryptophane, cysteine HCl, and adenine are dissolved in a little N HCl, and the xanthine, guanine, and uracil in a little N NaOH and then added to the filtrate. This solution is stored under toluene in the cold. It keeps for at least three months.

Salt Solution B

MgSO ₄ ·7H ₂ O	...	...	10 g.
Ferrous ammonium sulphate	...	...	0.7 g.
MnSO ₄ ·4H ₂ O	...	...	2 g.
Concentrated HCl	...	...	1 ml.
Distilled water to	...	...	250 ml.

"Vitamin" Solution C

Aneurine HCl	...	...	5 mg.
Pyridoxin	...	...	10 mg.
Pyridoxal	...	...	5 mg.
Pyridoxamine	...	...	5 mg.
p-Amino benzoic acid	...	...	1 mg.
Riboflavin	...	...	5 mg.
Nicotinic acid	...	...	5 mg.
Calcium pantothenate	...	...	5 mg.
Pteroyl glutamic acid	...	...	5 mg.
Distilled water	...	...	20 ml.

This solution is ampouled in 1 ml. amounts and stored in cold.

The complete basal medium (5/3 final strength) consists of:

Solution A	...	...	200 ml.
"Vitamin" solution C	...	...	1 ml.
			ampoule
Glucose	...	...	10 g.
Thiomalic acid	...	...	250 mg.
Guanylic acid, Na salt	...	...	62.5 mg.
Tween 80	...	...	0.5 ml.
Sodium phenobarbitone	...	...	0.5 g.
Biotin, solution of 1 μ g./ml. (B.D.H.)	...	...	2 ml.
Distilled water to final volume of	...	...	300 ml.

The glucose, guanylic acid, and sodium phenobarbitone are dissolved in sufficient water and added to the mixture before the medium is made up to the final volume of 300 ml. This amount of medium is sufficient for the assay of five sera together with a full range of standard B_{12} solutions and a control normal serum.

No pre-treatment of the casein hydrolysate (Allen and Hanbury acid hydrolysed casein for microbiological assay) as carried out by Spray (personal communication) was necessary. By accident, it was also found that adjustment of the pH of the basal medium to 5.5 was unnecessary, as the addition of 10 ml. of the cyanide-acetate buffer (pH 4.5)-treated solutions brought the pH of the final medium to 5.8.

Stock Cultures.—The lactobacillus was first grown in 5 ml. single strength basal medium to which was added 1% soluble proteolysed liver. After overnight incubation and centrifugation the greater part of the supernatant was discarded and the bacterial deposit resuspended in the remaining medium. This was then transferred to sterile ampoules to provide reserve stock cultures. Cultures for use are kept on solid media made from the proteolysed liver medium to which has been added 1% New Zealand agar. Such cultures remain viable for 14 to 21 days at 4° C. The advantage of using a solid medium is that a careful check can be kept on the purity of the strain.

Preparation of the Inoculum.—A single colony from the proteolysed liver agar plate is inoculated into 5 ml. proteolysed liver fluid medium. After overnight (18 hr.) incubation the culture is centrifuged and the supernatant discarded. The bacterial deposit is washed three times in 5 ml. sterile distilled water and finally suspended in 5 ml. sterile distilled water. Then 0.1 ml. of the suspension is inoculated into 5 ml. single strength basal medium and incubated anaerobically overnight (18 hr.), when 0.1 ml. of the undiluted overnight culture is then inoculated into 2 by 5 ml. of the same medium and incubated anaerobically for six hours. The resulting culture provides the inoculum for the assay. Using this technique an

even inoculum is obtained with no clumping of the organisms, and there are no irregularities in growth due to the carry-over of adsorbed B_{12} .

Preparation of Serum Extracts and Standard B_{12} Solutions.—To 1 or 2 ml. samples of serum measured with bulb pipettes is added from a burette 19 or 18 ml. of a mixture of NaCN and acetate buffer (0.1% NaCN 20 ml., 0.4 M sodium acetate buffer pH 4.5 50 ml., distilled water to 1,000 ml.) and the mixture is autoclaved at 5 lb. for 30 min.

A stock solution of B_{12} is made up by carefully measuring out with a tuberculin syringe 1 ml. of cytochrome 50 μ g./ml. (Glaxo) into a litre volumetric flask. This concentrated solution will keep without noticeable deterioration for three months in the ice-chest. From this stock new standard B_{12} solutions containing 25, 50, 100, 200, 400, and 800 μ g./ml. are dispensed for each assay run. Amounts, each of 1 ml., of these standard solutions are then treated in the same manner as the samples of serum. This method of preparing the standard solutions makes for greater accuracy, as a sterile technique is not necessary.

Assay Technique.—Amounts of basal medium, each of 15 ml., are dispensed from a burette into 6 by 1 in. "pyrex" test-tubes, capped with aluminium

TABLE I
 B_{12} LEVELS OBTAINED IN REPLICATE ASSAYS CARRIED OUT AT INTERVALS USING *LACTOBACILLUS LEISHMANII*

Date	Serum	Replicate Values (μ g./ml.)	Average Value (μ g./ml.)
July, 1956	Normal standard	260	270
	Patient R.	280	
		1,050	
Dec., 1956		1,000	1,000
	Normal standard	950	
	Patient L.	300	
	" T.	270	
		300	
Jan., 1957		110	285
	Normal standard	120	
	Patient E.	300	
	" W.	310	
		350	
Feb., 1957		25	33
	Normal standard	40	
	Patient Wh.	275	
		325	
		15	
Mar., 1957		25	20
	Normal standard	310	
	Patient K.	310	
		65	
		65	
May, 1957	Normal standard	300	310
	Patient P.	320	
		100	
		110	

Normal standard = 301 ± 21.5 .
Normal standard value as determined by an independent laboratory was 310 μ g./ml.

caps, and autoclaved at 10 lb. for 6 min. To tubes of this medium is then added 10 ml. of either the supernatant obtained from autoclaving the sera under

examination, or that obtained from a standard normal serum, or a similarly treated standard B₁₂ solution. All the sera are run in duplicate or triplicate. To the now completed medium is added 0.5 ml. of the inoculating culture and the whole shaken well to ensure even mixing. Four 5 ml. samples of the inoculated medium are then transferred (using sterile bulb pipettes) to sterile 6 by $\frac{5}{8}$ in. "pyrex" test-tubes, serially numbered and capped with coloured aluminium caps for identification. Incubation is carried out in a McIntosh and Fildes anaerobic jar simply evacuated with a water-pump and filled with hydrogen. This method of incubation was found to give not only the greatest growth in unit time (36 hr.) but also the most consistent growth in replicate assays. After incubation the density of growth is determined turbidimetrically using a nephelometer (E.E.L.), and from the results obtained with known amounts of B₁₂ (25, 50, 100, 200, 400, and 800 $\mu\mu\text{g.}/\text{ml.}$) a standard curve drawn.

Results

Using this technique it has been possible to obtain reproducible results with replicate samples of serum and repeated assays of a control normal serum (Table I).

Discussion

A complete assay, starting from the making of a fresh batch of medium to the obtaining of a final result, can be carried out within a working week. This seems to be of value when it is remembered that the technique described is designed only for the occasional assay. The drawback to occasional determinations, that of irregular results, is eliminated as shown by the results quoted. These determinations all necessitated a completely fresh start.

REFERENCE

Spray, G. H. (1955). *Clin. Sci.*, **14**, 661.