

## Microbiological Assay of Vitamins

### 1—THIAMINE

By

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For the microbiological assay of thiamine *SACCHAROMYCES CEREVISIAE* (1) and *LACTOBACILLUS FERMENTI* 36 (2, 3, 4, 5) have been chiefly employed. The earliest workers to use *LACTOBACILLUS FERMENTI* were Sarett and Cheldelin (2) who determined the response by photoelectric measurement of turbidity. Their basal medium and inoculation technique were subsequently modified by several investigators. Barton-Wright (3), Bennett and Kornberg (4), and Fitzgerald and Hughes (5). It is however significant that although thiamine has been assayed chiefly by the thiochrome reaction, microbiological methods have been widely used in the assay of the other members of the B-complex. Two possible reasons militating against the wider use of the microbiological technique for thiamine are the choice of a suitable basal medium and the difficulties inherent in turbidimetric measurements.

We have developed a technique employing a basal medium, which is not very different from that of Fitzgerald and Hughes (5) except for certain modifications on the lines suggested by Rabinowitz, Mondy and Snell (6) for *L. CASEI* and other actic acid bacteria in general. We have adopted the inoculation technique advocated by Barton-Wright (3) in preference to that employed by Cheldelin, Bennett and Kornberg (4) and Fitzgerald and Hughes (5). With our basal medium we have obtained quite satisfactory linear response for thiamine levels ranging from 0 to 0.05 micrograms per tube (10 ml.).

### Experimental

*Organism* *L. FERMENTI* 36, obtained from the National Collection of Type Cultures, Central Public Health Laboratory, London, was maintained by fortnightly sub-culture on a marmite-dextrose-agar supplemented with additional thiamine to an extent of 10 µg per tube of medium. After transfer the culture tubes were incubated at 37 deg. C. for 22-24 hours and then kept in the refrigerator. The stab medium was made up as follows:—

Marmite	..	..	1.5 g
Dextrose (A.R.)	..	..	0.5 g
Agar	..	..	2.0 g
Peptone (Gurr)	..	..	0.25 g
Salt Solution same as in basal medium	..	..	4.0 ml.
Thiamine HCl	..	..	1.0 mg.

The pH was adjusted to 6.5 and volume made up to 100 ml. The suspension was steamed to dissolve the agar. 10 ml. portions of the hot solution were measured into tubes, plugged with cotton wool and sterilised at 10 lb. steam pressure for 10 minutes. The tubes were allowed to cool until the agar set and then stored in a refrigerator.

### Basal Medium

				<i>Amount for 100ml. double strength medium</i>
Casein, tryptic digest	..	..	..	1000 mg.
Casein, acid hydrolysate	..	..	..	1000 mg.
Glycine	..	..	..	40 mg.
l-cystine	..	..	..	40 mg.
l-asparagine	..	..	..	20 mg.
dl-tryptophane	..	..	..	20 mg.
Adenine	..	..	..	2 mg.
Guanine	..	..	..	2 mg.
Uracil	..	..	..	2 mg.
Dextrose	..	..	..	4000 mg.
Sodium acetate, anhydrous	..	..	..	1200 mg.
NaCl	..	..	..	1000 mg.
KH <sub>2</sub> PO <sub>4</sub>	..	..	..	100 mg.
K <sub>2</sub> HPO <sub>2</sub>	..	..	..	100 mg.
MgSO <sub>4</sub> .7H <sub>2</sub> O	..	..	..	40 mg.
MnSO <sub>4</sub> .4H <sub>2</sub> O	..	..	..	2 mg.
FeCl <sub>3</sub>	..	..	..	0.4 mg.
Riboflavin	..	..	..	80 µg.
Niacin	..	..	..	80 µg.
Pyridoxin HCl	..	..	..	160 µg.
Calcium pantothenate	..	..	..	80 µg.
p-Amino benzoic acid	..	..	..	40 µg.
Folic acid	..	..	..	2 µg.
Biotin	..	..	..	0.4 µg.

The tryptic digest of casein was prepared according to Roberts and Snell (7) with certain modifications. 60 g. white soluble casein (B.D.H.) were dissolved by gradual addition with shaking to 1 litre of 0.8% sodium bicarbonate solution. To the uniform suspension thus obtained were added 300 mg. pancreatin (B.D.H.) suspended in 10 ml. of distilled water. The mixture was covered with a thin layer of toluene, shaken well and allowed to stand at 37 deg. C. for 48 hours. At the end of this period it was heated in flowing steam for 30 minutes to remove the toluene. After cooling to room temperature the pH was adjusted to 6.0 with glacial acetic acid and filtered with mild suction. To the filtrate 30 g. of activated charcoal (Merck) were added and shaken for 30 minutes. The resulting suspension was filtered and the filtrate was adjusted to pH 1.5 with concentrated HCl. This was then shaken with 30 g. Fullers earth (B.D.H.) for 30 minutes. The suspension was centrifuged and the supernatant decanted off. The pH of the supernatant was adjusted to 3.8 with 40% NaOH. This was shaken for 30 minutes with 12 g. of

activated charcoal (Merck). On filtering a clear, colourless liquid was obtained which was made up to 1200 ml. with distilled water and stored under toluene in a refrigerator.

The acid hydrolysed casein was prepared according to Snell and Rannefeld (8) with some modifications. 50 g. white soluble casein (B.D.H.) were refluxed for 10 hours with 500 ml. of 20% (w/v) hydrochloric acid. The excess hydrochloric acid was removed by concentrating to a thick syrup under reduced pressure. The syrup was dissolved in water and the distillation was repeated. The pH of the residue was adjusted to 1.5 with 40% (w/v) sodium hydroxide and the resulting solution was shaken with 10 g. Fullers earth for one hour and left overnight. It was then centrifuged to remove the earth and the supernatant was adjusted to pH 7 with 40% sodium hydroxide. The volume was made up to 500 ml. and shaken for 30 minutes with 5 g. activated charcoal. This was filtered and the charcoal treatment repeated twice. A clear, colourless hydrolysate was thus obtained and was stored under toluene in a refrigerator.

*Stock Solutions.*—The Amino acids were kept in a stock solution made up as follows:—2 g. glycine, 2 g. l-cystine, 1 g. dl-tryptophane and 1 g. l-asparagine were suspended in about 300 ml. distilled water, warmed up to 70-80 deg. C. and concentrated HCl added dropwise until a clear solution was obtained. This was cooled to room temperature, made up to 500 ml. with distilled water and stored under toluene at room temperature. This solution contains 4 mg. each of glycine and l-cystine and 2 mg. each of l-asparagine and dl-tryptophane per ml.

The purines were kept in stock solution made up as follows:—200 mg. each of adenine, guanine and uracil were suspended in about 150 ml. distilled water in a 200 ml. volumetric flask. 1 ml. of concentrated HCl was added and the solution was warmed to obtain a clear solution. The solution was cooled and volume made up to the mark with distilled water and stored under toluene at room temperature. This solution contains 1 mg. each of adenine, guanine and uracil per ml.

The salt solution which included dextrose and inorganic salts was prepared by dissolving them in suitable quantities in distilled water and was kept frozen at 4 deg. C. in the freezing chamber of a refrigerator.

Stock solution A of thiamine was prepared as follows:—50 mg. thiamine hydrochloride, which was dried in vacuo over phosphorus pentoxide for 48 hours, were accurately weighed, transferred to a 500 ml. volumetric flask and made up to the mark with 20% ethanol and 1 drop of concentrated HCl. This was stored under toluene in a refrigerator. This solution containing 100 µg thiamine/ml. keeps indefinitely.

Stock solution B of thiamine was prepared by transferring 1 ml. of stock solution A into a 100 ml. volumetric flask and making up to the mark with 20% ethanol and a drop of concentrated HCl. This was stored under toluene in a refrigerator and contains 1 µg thiamine per ml. It can be kept for a week.

*Standard solution of thiamine.*—1 ml. of stock solution B was accurately diluted to 100 ml. with distilled water. This solution is prepared only on the day of use and contains 0.01 µg thiamine per ml.

*Inoculum.*—The medium for growing the inoculum was conveniently prepared in 10 ml. centrifuge tubes. Into each of the centrifuge tubes plugged with cotton wool was measured 5 ml. of the double strength basal medium and 5 ml. of a solution of thiamine containing 2  $\mu\text{g}/\text{ml}$ . so that each tube will receive 10  $\mu\text{g}$  thiamine. The tubes were sterilised at 10 lb. steam pressure for 10 minutes. The organism was transferred from a stab culture to one of these tubes and incubated for 37 deg. C. for 22-24 hours. The tube was then centrifuged aseptically, the supernatant decanted off and the bacterial cells suspended in 10 ml. of sterile saline. This was centrifuged again, the supernatant decanted and the bacterial cells resuspended in 10 ml. of sterile saline. For the final inoculum 1 ml. of this suspension was diluted with 15 ml. sterile saline.

*Extraction.* The material to be tested was first homogenised. Plant materials and animal tissues were conveniently homogenised in a Waring blender while cereals and pulses were reduced to a fine powder in a grinder. The homogenised sample was suspended in 0.1 N sulphuric acid (about 15 times the weight of the material). This was digested on a steam bath for 30 minutes and allowed to cool. The pH was adjusted to 4.5 with sodium hydroxide. 20 mg. of white papain (obtained locally) and 20 mg. takadiastase (Parke Davis & Co.) for every gramme material used were added and incubated at 37 deg. C. for 24 hours under a thin layer of toluene. This was steamed for 30 minutes, allowed to cool, pH adjusted to 6.5 with sodium hydroxide and filtered through Whatman No. 1 filter paper. An aliquot was suitably diluted so that it contained approximately 0.005 to 0.01  $\mu\text{g}$  thiamine per ml.

*Assay procedure.* Pyrex lipless test tubes, 16  $\times$  125 mm., were used for the assay. Into 16 tubes were measured 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 ml. of the standard solution of thiamine in duplicate. The volume in each tube was made up to 5 ml. with distilled water. 5 ml. of double strength thiamine free basal medium were added to each tube. Into 8 other tubes were measured 1, 2, 3 and 4 ml. of the test extract in duplicate. As before, the volume in each tube was made up to 5 ml. with distilled water followed by addition of 5 ml. of double strength thiamine free basal medium. The tubes were plugged with cotton wool and sterilised at 10 lb. steam pressure for 10 minutes.

On cooling to room temperature, each tube was inoculated with one drop of the diluted inoculum by means of a pasteur pipette. The tubes were incubated at 37 deg. C. in a constant temperature water bath for 17-18 hours. They were then chilled in the refrigerator for 15 minutes to arrest further growth of the microorganism, warmed to room temperature (28 deg. C.) and the turbidities measured in a Klett-Summerson photoelectric colorimeter, using green filter 54 (range 520-580 m $\mu$ ).

*Results.* The thiamine content of the sample is deduced by the 'slope-ratio method' (9, 10). Example

Volume of standard thiamine solution (ml).	Corresponding wt. of thiamine ( $\mu$ g).	Mean turbidity values.
0	0	0
0.5	0.005	4
1.0	0.010	10
1.5	0.015	17
2	0.020	26
3	0.030	37
4	0.040	55
5	0.050	75

The equation of the 'best line' through these points is calculated as follows:—  
suppose  $x$  is the dose of thiamine and  $y$  the corresponding turbidity value (response

$x$	$y$	$x^2$	$y^2$	$x - m_x$	$y - m_y$	$(x - m_x)(y - m_y)$	
0	0	0	0	-0.021	-28	0.588	
0.005	4	0.000025	16	-0.016	-24	0.384	
0.010	10	0.000100	100	-0.011	-18	0.198	
0.015	17	0.000225	289	-0.006	-11	0.066	
0.020	26	0.000400	676	-0.001	-2	0.002	
0.030	37	0.000900	1369	0.009	9	0.081	
0.040	55	0.001600	3025	0.019	27	0.513	
0.050	75	0.002500	5625	0.029	47	1.363	
Total	0.170	224	0.005750	11100	0	0	3.195
Mean	0.021	28	0.000719	1388			0.3994
	$m_x$	$m_y$	$m_x^2$	$m_y^2$			
	$x$	$y$	$x^2$	$y^2$			

From the above table the correlation coefficient  $r$  may be calculated by means of the formula.

$$r = \frac{\text{mean of the product } (x - m_x)(y - m_y)}{\sigma_x \times \sigma_y}$$

$$\text{where } \sigma_x = \text{standard deviation of } x = \sqrt{m_x^2 - (m_x)^2}$$

$$\sigma_y = \text{standard deviation of } y = \sqrt{m_y^2 - (m_y)^2}$$

In this example,

$$r = \frac{0.3994}{\sqrt{\{0.000719 - (0.021)^2\} \{1388 - 28^2\}}}$$

i.e.  $r = 0.975$ . When  $r = 1$ , there is complete dependence of the response on the dose. For other values of  $r$  between 0 and 1, the degree of dependence is low as it approaches 0 and high as it approaches 1.

The equation of the 'best line' is calculated by means of the formula.

$$x - m_x = r \cdot \frac{\sigma_x}{\sigma_y} \cdot (y - m_y)$$

Hence for this example the equation becomes

$$x - 0.021 = 0.975 \sqrt{\frac{0.000719 - (0.021)^2}{(1388 - 28^2)}} \times (y - 28)$$

$$\text{i.e. } y = 1512x - 3.8$$

Similarly the correlation coefficient and equation of the 'best line' for the test extract can be calculated. For example for a specimen of 'Molagusamba'—a variety of pure line paddy grown in Ceylon.

Vol. of test extract (x)	0	1	2	3	4	ml.
mean turbidity values (y)	0	10	20	35	50	
$r$	= 0.994					
$y$	= $12.64x - 2.3$					

If the assay is perfectly valid, theoretically, the standard and test lines should intersect on the vertical axis of response. In practice, however, small divergences may occur but can be disregarded as long as the points of intersection lie very close to each other.

The slope of the standard line is 1512 units turbidity/ $\mu$ g thiamine. The slope of the test line is 12.64 units turbidity/ml. extract. Hence thiamine content of extract is  $\frac{12.64 \mu\text{g}}{1512}$  ml. extract.

$$\text{or } \frac{12.64 \times 5 \times 10^4 \mu\text{g}/100 \text{ g.}}{1512}, \text{ since } 5 \times 10^4 \text{ is}$$

the dilution factor in this particular example. Hence thiamine content in 100 g. of 'Molagusamba' is 418  $\mu$ g.

### Discussion

In an earlier basal medium the concentrations of NaCl and glucose we employed were 1 mg. and 1 g. per 100 ml. respectively—concentrations employed by Rabinowitz, Mondy and Snell (6) for *LACTOBACILLUS CASEI* and recommended for other lactic acid bacteria as well. When *L. FERMENTI* was grown in this medium, instead of an even suspension of cells, there was a marked tendency for the cells to clump not only in the inoculum medium but also in the assay tubes making it difficult for reliable turbidimetric measurements. When the cells in the inoculum were centrifuged and resuspended in isotonic saline, they tended to flocculate. On increasing the concentrations in the basal medium of NaCl and glucose to 0.5 g. and 2 g. per 100 ml. respectively as recommended by Barton-Wright (3) we com-

pletely overcame this difficulty. We mention this because Barton-Wright did not give any special reasons for altering the concentrations of NaCl and glucose.

At first we followed the inoculation technique described by Cheldelin, Bennett and Kornberg (4) and Fitzgerald and Hughes (5) according to which one drop of the saline suspension of the centrifuged cells was diluted in 10 to 25 ml. of sterile saline. With this procedure we obtained very inconsistent results even with the standard solution of thiamine at different levels. Duplicates also gave widely differing turbidity values. Therefore we adopted Barton-Wright's technique of diluting 1 ml. of the cell suspension in 15 ml. of sterile saline and we succeeded in obtaining not only good agreement between duplicates but also very low blank values. We attribute our low blank values to our method of subjecting the tryptic digest and the acid hydrolysate of casein to fullers earth treatment in addition to the usual activated charcoal treatment.

We have experienced no difficulty in subculturing *L. FERMENTI* on marmite media the composition of which has been described elsewhere in this paper.

### Summary

- (1) A method for the microbiological assay of thiamine is described.
- (2) Modifications have been made in the composition of the basal medium.

### References

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