A Microbiological Assay of Vitamin B₁

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A microbiological assay for thiamine, using the yeast *Kloeckera brevis* is described. The method is simple and specific. The sensitivity is high enough to allow the determination of 0.001 μ g of thiamine per ml of extract with a standard deviation of less than 10 %.

Although a large number of methods for the determination of thiamine (vit. B₁) has been described, each has certain limitations.

Modifications of Jansens¹ fluorometric thiochrome method seems to be preferred in most laboratories, although this method is neither as specific nor as sensitive as the microbiological methods.

The only microbiological assay which seems to have been generally used is modifications² of the *Lactobacillus fermentum* method, originally described by Sarett and Cheldelin³. *L. fermentum*, however, is very exacting and some laboratories including our own have been unable to obtain consistent satisfactory results with this organism.

We have therefore investigated a number of thiamine requiring micro-organisms in search of a less exacting strain which could be used both for the routine analysis of thiamine in natural products and in an investigation of the effect of various factors on the destruction of this vitamin. Of the organisms tested *Kloeckera brevis* gave the most promising results. Burkholder⁴ has shown that this yeast strain requires six members of the B-vitamin group for optimal growth and he has recommended this organism for the assay of inositol. Our preliminary tests, however, showed that none of the media described for *Kloeckera brevis* allow maximum growth in a thiamine assay. The main problem therefore was to devise assay conditions under which thiamine is the only limiting factor for growth.

METHOD

a. *Organism*. *Kloeckera brevis* B 768, obtained from *The National Collection of Industrial Bacteria*, Teddington, Middlesex, U.K.

b. *Agar medium*. Dissolve 20 g of malt extract, to which no preservative has been added, in 200 ml of water. Adjust the pH to 5.0 and dissolve 5.0 g of agar in the mixture at about 100° C. Dispense the medium in 5 ml quantities to test tubes. Plug

the tubes with cotton, autoclave at 120° C for 15 min., slope, and store in a refrigerator. After transfer incubate the cultures at 30° C for 16–24 h and keep them in a refrigerator until the next transfer. Transfer the stock cultures at monthly intervals.

c. *Inoculum*. Remove about 2 mg of wet yeast cells with a bacteriological loop from a 16–24 h old culture and suspend in 10 ml of sterile tap water. Dilute 1 ml of this suspension with 10 ml of sterile tap water. Use 0.05 ml of this diluted cell suspension for the inoculation of each tube.

d. *Basal medium*.

0.5 g	casamino acids (Difco)
4.0 g	glucose
10 ml	salt E
5	» trisodium citrate · 5 H ₂ O solution, 15 % w/v
2	» L-asparagine solution, 2 % w/v
5	» yeast extract solution
1	» vitamin solution

Mix the solutions, add 50 ml of water and dissolve the glucose and the casamino acids in the mixture. Adjust the pH to 4.7 with 1 N HCl and add water to make 100 ml.

Prepare the solutions as follows:

Salt E. Dissolve 22 g KH₂PO₄, 17 g KCl, 10 g (NH₄)₂SO₄, 4 g CaCl₂, 4 g MgSO₄ · 7 H₂O, 0.1 g FeCl₃, 0.1 g MnSO₄ · 4 H₂O and 10 ml conc. HCl in water to make 1 000 ml.

Vitamin solution. Dissolve 100 mg meso-inositol, 20 mg choline chloride, 50 µg (+) biotin and 5 mg each of nicotinic acid; pyridoxine chloride and calcium pantothenate in water to make 50 ml. Store under a preservative in a refrigerator. Prepare a fresh solution every month.

Yeast extract solution. Dissolve 5 g of yeast extract (Difco) and 0.25 g of Na₂S₂O₃ · 7 H₂O in 50 ml of water, adjust the pH to 5.4 and autoclave the mixture at 120° C for 30 min. Cool the solution and add an exact amount of 2 % H₂O₂ solution (about 6 ml) to oxidize excess sulfite. A freshly mixed solution of equal parts of 5 % KI solution, 1 % starch solution and 50 % w/v sulfuric acid solution is used as an external indicator. Dilute the yeast extract solution with water to make 100 ml. Store under a preservative in a refrigerator. Prepare a fresh solution every month.

Standard thiamine solution, 0.0025 µg per ml. Dry thiamine dichloride (M 337.3) over H₂SO₄ in vacuum to constant weight.

Stock solution: Dissolve 100 mg of the dried vitamin in 50 ml of 0.1 N HCl and add water to make 100 ml. Store in a refrigerator. Prepare a fresh solution every month. The standard solution is prepared fresh every day by diluting 250 µl (constriction pipette) of the stock solution with water to make 100 ml.

e. *Assay procedure*. The assay is carried out in lipless uniform test tubes (100 × 16 mm. i.d.). To each series the standard thiamine solution (2.5 mµg per ml) is added in the following amounts: 0, 0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 ml, each with an error of not more than 2 %. Each level is set up in triplicate. The sample to be assayed is similarly added to a series of tubes in the following amounts: 0.3, 0.5, and 0.8 ml in duplicate. Water is added to the tubes to make a total volume of 1 ml, and 0.95 ml of the basal medium is added. The tubes are shaken to mix the content, covered with a cloth, steamed at 100° C for 10 min., cooled and inoculated. In each set of standard tubes one of the tubes containing 0 ml of standard solution is left uninoculated. These tubes are used as blanks in the turbidimetric determination of growth. The tubes are incubated at 30° ± 0.5° C for 18–24 h in a *shaking machine* to guarantee sufficient aeration and uniform growth.

f. *Determination of response*. The tubes are shaken and the cultures transferred to a microcuvette. The turbidity is read at $\lambda = ca. 650 m\mu$ in a suitable photometer (e.g. Lumetron 402 E).

g. *Calculation of results*. A standard dose-response curve is prepared by plotting the average of the turbidity values found at each level of the thiamine standard against the amount of thiamine present (Cf. Table 1 and Fig. 1). The vitamin content of a sample is determined by interpolating the response to the known amounts of the test solution onto this standard curve. The thiamine content per ml of the test solution is now calculated for each duplicate sets of tubes, and the thiamine content of the sample is calculated from the average of the values.

h. *Preparation of samples for assay.* Enough of the material to give a representative sample is suspended in at least 25 ml of 0.1 *N* sulfuric acid per g dry matter. The mixture is steamed for 30 min. at 100° C and the pH adjusted to 4.7 with 2.5 *M* sodium acetate. 100 mg of mylase P (Wallerstein Lab., N.Y.) or another suitable enzyme preparation is added per g of dry matter. The mixture is covered with a preservative and incubated at 37° C for 18–24 h. The digest is then steamed for 10 min. at 100° C and diluted with water to a suitable volume. After filtering through filterpaper the clear filtrate is diluted to contain 1–2 μg of thiamine per ml. The content of thiamine in each batch of enzyme preparation must be determined and if significant taken into account.

i. *Reproducibility of the assay.* The standard deviation of repeated independent assays of several crude materials has been 5–8 %.

RESULTS

a. *Effect of yeast extract on growth.* For some time we used the basal medium described without the addition of the sulfite treated yeast extract after having convinced ourselves that neither the addition of other substances known to be growth factors for microorganisms (vitamins, purines, pyrimidines, nucleotides, deoxynucleotides, salts, amino acids etc.) nor the addition of alkali-treated peptone, malt extract and yeast extract enhanced the growth. Sometimes, however, assays of crude material showed positive "drift" and occasionally results seem to be too high. As shown by Williams⁵ sulfite under rather mild conditions destroys the thiamine activity by splitting the thiamine molecule. We therefore tested the growth effect of some sulfite-treated natural products and found that yeast extract and malt extract in which the thiamine was destroyed by sulfite did actually enhance the growth (Fig. 1). After the addition of an optimal amount of sulfite-treated yeast extract thiamine seems to be the only limiting factor for growth as no "drift" occurred in the assay of natural products as exemplified in Table 1.

Fig. 1. Growth curves for *Kloeckera brevis*. I Medium as described.
II Medium as described without yeast extract.

Table 1. Determination of thiamine in beef. 5 g of minced beef extracted as described. Final solution ~ 1.25 mg beef per ml. Total volume: 2 ml per tube.

Standard					Beef extract				
Thiamine	Turbidity				Beef	Turbidity			Thiamine
mμg per tube	ser. 1	ser. 2	ser. 3	mean	mg per tube	ser. 1	ser. 2	mean	mμg/mg beef
0	90	89	90	90	0.125	82	80	81	1.12
0.25	75	77	76	76	0.25	72	71	72	1.20
0.5	63	64	62	63	0.50	63	61	62	1.02
1.0	42	(90)	42	42	0.75	44	44	44	1.22
1.5	29	30	28	29	1.00	39	37	38	1.12
2.0	21	19	20	20	1.25	32	(12)	31	1.05
2.5	(21)	14	15	15					
						mean:			1.12

b. *Response to cocarboxylase and to the pyrimidine and thiazole moieties of thiamine.* On a molar basis cocarboxylase (thiaminepyrophosphate) has about 30 % less growth promoting activity as thiamine. After treatment with mylase, as described for the preparation of samples for assay, the response to cocarboxylase is the same as to thiamine.

For several microorganisms the growth effect of thiamine can be substituted by either both or one of the two main fission products of thiamine namely, 2-methyl-4-amino-5-aminomethyl-pyrimidinedichloride and 4-methyl-5-(β-hydroxyethyl)thiazole. For mammals these two naturally occurring substances are without vitamin activity. The same holds true for *Kloeckera brevis*. Even after incubation for 72 h neither of the two substances nor a mixture of both can substitute thiamine for growth. At least up to concentrations 10 times the maximum concentration of thiamine used in the assay, they have no inhibitory effect either.

Table 2. Results of analysis of some products for their thiamine content by two methods.

Product	Thiamine μg/g	
	<i>Kloeckera brevis</i> method	Thiochrome method
Wheat flour	1.85	1.65
» » vitaminized	5.60	5.25
Rye bread	2.20	2.30
Fodder additive A	58.5	58.0
Liquor B-vitaminorum	48.0	45.5
Fodder yeast 1.	1.90	1.75
» » 2.	2.70	2.45
» » 3.	11.5	12.0
Fodder additive B	110	115

c. Comparison of results obtained by the Kloeckera brevis method and the thiochrome method.

As seen from Table 2 the results of the two methods agree reasonably well.

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