MICROBIOLOGICAL ASSAY FOR THIAMIN USING *LACTOBACILLUS VIRIDESCENS*[1]

ROBERT H. DEIBEL, JAMES B. EVANS, AND C. F. NIVEN, JR.

Division of Bacteriology, American Meat Institute Foundation, The University of Chicago, Chicago, Illinois

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Thiamin is one of the few vitamins for which analytical laboratories generally employ a chemical rather than a microbiological method for quantitative assay (Association of Vitamin Chemists, 1950). A wide variety of microorganisms have a nutritional requirement for thiamin and numerous organisms have been proposed for the microbiological determination of this vitamin (Barton-Wright, 1952; Hoff-Jorgensen and Hansen, 1955). However, each of these proposed methods suffers from one or more disadvantages such as lack of specificity, interference by inhibitory substances, nonspecific stimulatory substances, or complexity of medium and procedure.

It has been reported (Deibel *et al.*, 1955, 1957) that *Lactobacillus viridescens* (Niven and Evans, 1957) has an unusually high requirement for thiamin in a medium containing tryptone and thiamin deficient yeast extract. This report presents a method for thiamin assay using *L. viridescens* that is more specific, more convenient, and less subject to interfering substances than other methods of thiamin assay that are currently available.

MATERIALS AND METHODS

Organism. The test organism, *L. viridescens* strain S38A, is now available from the American Type Culture Collection as no. 12706. It is a heterofermentative organism that grows very poorly or not at all in most commercially available media as a result of some rather unusual nutritional requirements (Evans and Niven, 1951; Deibel *et al.*, 1955).

Inoculum. The inoculum is grown at 30 C for 16 to 20 hr in APT[2] broth that has been prepared with yeast extract containing a sufficient amount of thiamin to give maximum growth of the test organism. The composition of APT broth is listed in table 1. The cells are centrifuged, resuspended and diluted in distilled water, and one drop added to each assay tube. Preliminary experiments have indicated that the washed cells may be resuspended and diluted in the assay basal medium, frozen and stored in the ice cube compartment of a refrigerator for as long as 30 days, thawed, and used directly to inoculate the assay tubes. This latter method may prove to be an added convenience in some laboratories.

Assay medium and procedure. Double strength APT broth is prepared with thiamin deficient yeast extract and the pH adjusted to 6.0. (Experimental lots of this assay medium have been prepared in dehydrated form by Difco Laboratories and these have proved to be satisfactory.) Five ml of the double strength medium are added to each assay tube, thiamin dilutions and appropriate dilutions of the extract from the sample that is to be assayed are added to the tubes, the volume brought to 10 ml in each tube with distilled water, and the tubes are heated in the autoclave for 5 min at 15 lb pressure. It has been found that at pH 6.0 there is no significant loss of thiamin during this heating. Not all bacterial spores are killed by this treatment, but the survivors do not germinate and grow rapidly enough to interfere with the assay.

Measurement of response. Growth is measured turbidimetrically (optical density at 660 mμ) in the 18.0 mm matched pyrex tubes used for the assay. The incubation period is 20 hr at 30 C. Incubation for more than 24 hr is not advisable because of the possible interference from spore-forming bacteria. It has also been found that titration values do not correlate well with turbidity readings or thiamin level.

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[2] The letters "APT" are an arbitrary designation having no abbreviative significance. This medium was first referred to by Evans and Niven (1951).

TABLE 1

*Composition of APT broth**

Compound	Amount
	g/L
Tryptone	10
Yeast extract	5
Sodium chloride	5
Sodium citrate	5
Dipotassium phosphate	5
Glucose	10
	ml/L
Salts C†	20
Tween 80‡	1

* pH 6.7–7.0.

† Contains per 100 ml: $MgSO_4 \cdot 7H_2O$, 4.0 g; $FeSO_4 \cdot 7H_2O$, mg; NaCl, 200 mg; and $MnCl_2 \cdot 4H_2O$, 720 mg.

‡ Polyoxyethylene sorbitan monooleate.

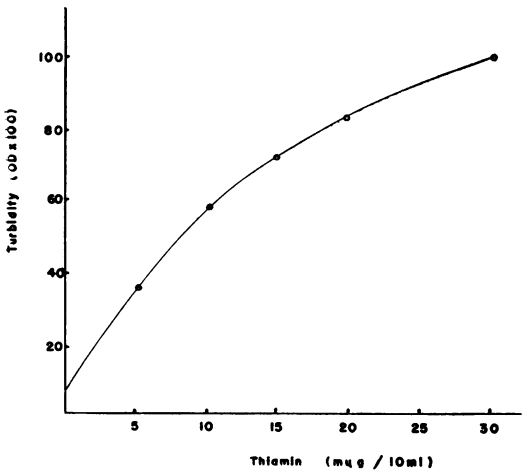

Figure 1. Growth response of *Lactobacillus viridescens* to thiamin added to thiamin-deficient APT broth.

RESULTS

A typical standard curve is given in figure 1. The growth response continues to increase at a fairly constant rate up to a thiamin level of 100 mμg per 10 ml which gives a turbidity value of 140 (optical density of 1.4).

Equimolar concentrations of cocarboxylase are approximately 60 per cent as active as thiamin, as shown in figure 2.

The pyrimidine and thiazole moieties of thiamin are not utilized by the test organism when added either singly or together. Furthermore, as shown in table 2, addition of as much as 15 μg of each moiety per tube had no effect on the response to the thiamin levels in the standard curve.

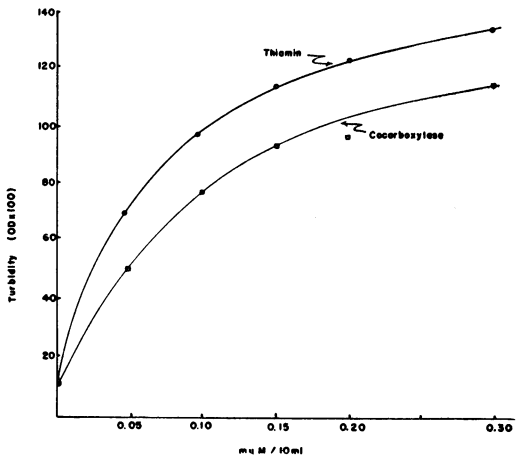

Figure 2. Comparative activity of thiamin and cocarboxylase for *Lactobacillus viridescens.*

TABLE 2

Growth response (optical density × 100) of Lactobacillus viridescens to thiamin in the presence of possible interfering substances

Added Substances*	Thiamin (mμg per 10 ml)					
	0	10	20	30	50	100
None	11	58	82	92	110	135
Moieties	8	60	82	92	115	135
Pentoses	14	60	80	96	115	130
Fructose	12	60	82	88	102	130
Maltose	11	60	77	83	96	135
Fructose + maltose	10	58	83	88	103	130
Calcium	12	62	85	96	110	140
Thioglycolate	12	60	85	95	110	140
Acetate	12	58	87	97	115	135

* These were added as follows: Moieties—15 μg of both the pyrimidine and thiazole moieties of thiamin; pentoses—50 mg of ribose, 50 mg of xylose, and 50 mg of arabinose; fructose, 50 mg; maltose, 50 mg; $CaCl_2 \cdot 2H_2O$, 2.2 mg; sodium thioglycolate, 2.5 mg; sodium acetate, 50 mg.

The standard response curve is also unaffected by reasonable levels of pentoses, fructose, maltose, calcium, thioglycolate, or acetate. These results are also summarized in table 2. All of these substances have been reported to affect the response of various other test organisms.

Extracts of several natural products have been assayed for thiamin using the *L. viridescens* assay

TABLE 3

Thiamin content of natural products as determined by the thiochrome and Lactobacillus viridescens methods

Method	Pork	Flour	Yeast Extract
	μg/g of sample		
Thiochrome	4.55	4.57	7.54
*L. viridescens**	4.3	4.3	7.5
	4.7	4.3	7.5
	4.3	4.3	7.9
	4.7	4.4	7.6

* The four values represent results on four different dilutions that gave a growth response within the more accurate range of the standard curve.

and the results compared with independent analyses of the same extracts by the thiochrome method. The results are summarized in table 3. The results compare quite favorably, and different dilutions of the same extract give values that are in good agreement. The extracts for these experiments were prepared by the standard method employed for the thiochrome analysis. There are some indications that less elaborate extraction procedures may be adequate for this microbiological assay. However, comparison of extraction procedures was considered to be beyond the scope of this investigation.

DISCUSSION

Hoff-Jorgensen and Hansen (1955) have pointed out that the two most common methods of thiamin analysis, the thiochrome method and the *Lactobacillus fermenti* method of Sarett and Cheldelin (1944), are both relatively unsatisfactory. The thiochrome method is not as specific nor as convenient as a suitable microbiological method. *Lactobacillus fermenti* assays are affected by pentoses (Camien and Dunn, 1955), reducing agents, fructose and maltose (Snell and Lewis, 1953), calcium (Yu and Sinnhuber, 1955), and heat degradation products of glucose (Ramsey and Lankford, 1956). The *Kloeckera brevis* method of Hoff-Jorgensen and Hansen is too new to evaluate completely, but it appears to be more cumbersome than the *L. viridescens* assay described here.

Other microorganisms that have been proposed by earlier workers for thiamin assay, and rejected by subsequent workers for a variety of reasons, are: *Staphylococcus aureus* (West and Wilson, 1938), *Saccharomyces cerevisiae* (Schultz *et al.*, 1942), *Phycomyces blakesleeanus* (Hamner *et al.*, 1943), and *Streptococcus salivarius* (Niven and Smiley, 1943).

L. viridescens is generally more specific, less sensitive, and more consistent in its response to added thiamin. The basal medium is relatively simple, and the inoculum is conveniently prepared. Thus, it is believed that the assay method using this organism will prove to have distinct advantages over previously described methods, both microbiological and chemical.

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SUMMARY

A microbiological method of thiamin assay using *Lactobacillus viridescens* is described. This method is convenient, rapid, relatively specific, and less subject to interfering substances as compared to previously described methods for determination of this vitamin. Cocarboxylase has about 60 per cent of the activity of equimolar quantities of thiamin. The pyrimidine and thiazole moieties of thiamin are not utilized, either individually or together, and the results are not affected by reasonable levels of pentoses, fructose, maltose, calcium, thioglycolate, or acetate.

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