Microbiological Activity of Some Mixed Disulfides of Thiamine for *Lactobacillus fermenti*

Z. G. BÁNHIDI

Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

Thiamine, its ortho-, and pyrophosphate form (as well as other phosphorylated forms) are equally active for growth promotion of *Lactobacillus fermenti* strain 36 (ATCC 9338) in the absence of cystine and cysteine. [1,2] Under these conditions, however, the homomeric disulfides of thiamine and its phosphorylated derivatives will not be utilized by the bacterial cells. Addition of cysteine, ascorbic acid and other reducing agents or reduction of the disulfides by heating is necessary for these substances to satisfy the absolute requirement of thiamine for *L. fermenti*. *

Our results obtained by growth analysis in living cells, were in full agreement with reports based on enzymatic model systems, in which the homomeric disulfide of thiamine pyrophosphate (cocarboxylase) was found to require activation by reduction for function as coenzyme during anoxidative or oxidative pyruvate-decarboxylation [3-5]. Both the data obtained with our growth test and those of the enzymatic model system must be considered as *per se* counter-evidences against a sulfhydryl-disulfide function of thiamine in the cells, according to

$$2\ T_{ol} \rightleftharpoons T\text{-}SS\text{-}T \quad \ldots\ldots\ldots\ a$$

or more correctly

$$2\ TPP_{ol} \rightleftharpoons TPP\text{-}SS \quad \ldots\ldots\ldots\ b$$

Nevertheless, none of these results exclude the probability that thiamine or its phosphates function as carriers of alien sulfhydryl compounds by forming heteromeric mixed disulfides in connection with some physiological process. Extended work on the biological effect of the mixed disulfides was justified because of the discovery of formation of several allithiamine derivatives in the garlic [6] and the report on the superiority of thiamine propyl disulfide toward thiamine in the treatment of some thiamine deficiency symptoms [7]. For the test, our growth system was used instead of enzymatic models, partly because the mixed disulfides of thiamine are more readily available than those of thiamine pyrophosphate, and because the utilization of a substituted nutrient by a homogeneous population of growing cells offers more general information than an isolated enzymatic model system, which has unfortunately not yet reached the desirable purity.

Experimental. The term »utilization» of some substances related to thiamine was intermittently used by us to denote the growth promoting ability of a compound in a defined medium. In this sense the compound in question was considered as a nutrient and not as an established metabolite. We tacitly assume, however, that the inability of the *L. fermenti* cells to grow on a certain thiamine derivative might depend on several different reasons, *e. g.*, on the cell wall barrier, or on the lack of enzymes

Table 1.

	Thiamine equivalent 2 μg			4 μg		
Thiamine *	40	42	43	36	35	35
T—SS—T	83	82	81	81	82	84
T—SS—T incubated with cell mash of *L. fermenti*	90	92	94	85	84	97
as above plus cysteine added	40	39	38	35	33	35
T—SS—T plus cysteine	38	38	50	38	37	39
T—SS—T incubated with intact cells of *L. fermenti*	90	90	91	87	86	91
as above plus cysteine added	66	93	34	47	59	42
The pH of incubation	*4.5*	*6.0*	*8.0*	*4.5*	*6.0*	*8.0*

* Numerical values are means of two turbidimetric readings at each concentration and are expressed as per cent of light transmitted at 5 950 Å.

* *Abbreviations* not explained in the legend of Fig. 1.: The thiol form of thiamine (or its derivatives) T_{ol}; the thiazole form of the same, T_{tz}; the homomeric disulfide of thiamine T—SS—T, and that of thiamine pyrophosphate TPP—SS, while cocarboxylase is denoted as TPP.

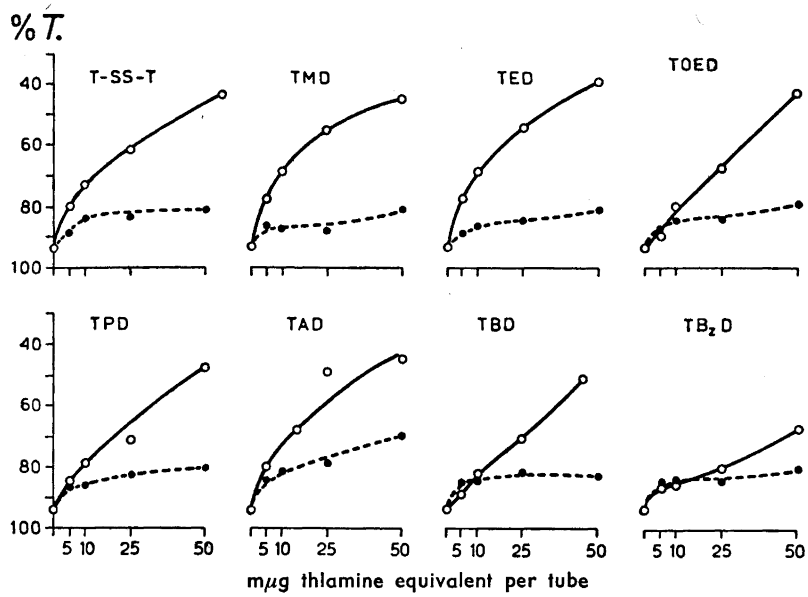

Fig. 1. Growth test of different disulfide derivatives of thiamine with *Lactobacillus fermenti* strain 36 on fluid medium. Abbreviations not occurring in the text are in accordance with the terminology proposed by Matsukawa [13], *viz.*, TMD, thiamine methyl disulfide; TED, thiamine ethyl disulfide; TOED, thiamine hydroxyethyl disulfide; TPD, thiamine propyl disulfide. TAD, thiamine allyl disulfide; TBD, thiamine butyl disulfide; TB_ZD, thiamine benzyl disulfide. Dotted lines represent test in absence of cysteine and full lines were used when 500 μg cysteine was added to each ml of the medium. All thiamin derivatives were added aseptically to the autoclaved medium in order to protect the disulfides from reduction. Growth was measured turbidimetrically in dublicates, and expressed as per cent transmittance (% *T.*).

able to convert the disulfides to a form available for thiaminokinases.

With these possibilities in mind we tried to split the homomeric disulfide of thiamine with the aid of cell free extracts of *L. fermenti* as well as with large amounts of living bacterial cells.

One day old culture of *L. fermenti*, grown on a complete medium (micro inoculum broth, Difco) was washed twice in saline, frozen in acetone-dry ice mixture and subjected to desintegration, according to Hughes [6]. Disruption of the cells was registered microscopically. Ten mg of the homogenized cell mash was Seitz-filtered and incubated with one mg of T—SS—T at 37°C for 30 min in 1/15 M phosphate buffer at different pH's, as seen in Table 1.

Intact washed cells were also subjected to incubation with T—SS—T for comparison. In each case 10 mg wet cell sediment was used as starting material and combined with 1 mg T—SS—T in 5 ml buffer solution. Similar batches were supplemented with 400 μg cysteine, for controls. The batches, containing intact cells, were Seitz-filtered after incubation in order to ensure aseptic test. Activity tests with *L. fermenti* were performed, according to former experiments on fluid media lacking cysteine [9,2] by using suitable dilutions of the above preparations. The results show that neither the cell mash nor the intact cells were able to liberate thiamine activity from T-SS-T under conditions where cysteine easily performed the activation of T—SS—T to growth factor for *L. fermenti*.

An enzymatic test which shows inability of cells or cell free preparations to perform an expected reaction must be considered cautiously since physical conditions might have been unfavorable during the experiment. In the present case, however, we are dealing with a substrate (T—SS—T) which is labile enough

at the conditions used (at least at slightly basic pH) to be easily converted to free thiamine as was shown by controls containing cysteine.

As an extension of the enzymatic work, *L. fermenti* was cultivated on the synthetic basal medium supplemented with thiamine sufficient for more than half-maximum growth. Cells were then harvested and washed twice by centrifuging and Seitz-filtration in order to make available the thiamine factors of the bacteria for analysis. Batches of the cell mash were chromatographed on paper in several solvent systems and afterwards developed bioautographically according to the procedure detailed in the subsequent paper [10]. The bioautographs of the bacterial cell mash revealed the presence of four distinct spots tentatively identified as thiamine pyrophosphate, thiamine orthophosphate, free thiamine-thiazole and a minor fraction (less than about one tenth of any of the former) behaving in all respect as T−SS−T.

An explanation for the contradiction between the cell mash experiment showing inability to split T−SS−T and the bioautographic analysis showing the presence of minute but distinct zones of T−SS−T in the cell mash, would be the theory that T−SS−T represents the end product of the thiamine/cocarboxylase metabolism.

In the hope of obtaining more information about the possible role of the disulfides of thiamine in the cells, experiments were performed with some mixed disulfides of thiamine.

The disulfides of thiamine subjected to microbial growth test were T−SS−T (prepared according to Zima *et al* [11] and seven different allithiamine homologues prepared by and obtained from Matsukawa [12,13]. Description of the compounds, explanation of abbreviations and the results obtained are given in Fig. 1. The conditions of the tube test for growth of *L. fermenti* on a chemically defined basal medium as well as details of cultivation of stock culture and the technique of turbidimetric readings were the same as in our earlier work [9,2]. Each substance was tested in both cysteine free medium and with added cysteine. Under these conditions thiamine was active in both cases giving quantitative results rather similar to that of T−SS−T with cysteine. (For this reasons thiamine values were left out from Fig. 1.) On the other hand only six of the thiamine alkyl disulfides supported the growth of *L. fermenti* to an extent comparable with T−SS−T on molar basis. All these derivatives required, however, activation by cysteine in a similar way as T−SS−T, demonstrating that the mixed disulfides of thiamine tested in this work, are not directly utilizable by the cells of *L. fermenti*. Thiamine benzyl disulfide showed very little increase in activity after addition of cysteine. This suggests the presence of a structural hindrance in the molecule for splitting the disulfide linkage with cysteine or for replacement of the benzylmercaptan with the same.

Conclusion. Intact or disrupted cells of *Lactobacillus fermenti* are probably not able to liberate thiamine from thiamine disulfide

Several different alkyldisulfides of thiamine behave similary to the homomeric disulfide of thiamine in a growth test with *L. fermenti* and serve as nutrient for the cells only after activation by cysteine or other reducing agents in contrast to thiamine, which supports growth without activation. Thiamine benzyl disulfide resists cysteine activation.

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