Thiamine Pantetheine Disulfide as a Microbial Growth Factor

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In connection with our study on the nutritional requirements of *Lactobacillus fermenti*, strain 36 (ATCC 9338), a growth enhancement with thiamine was observed, when yeast extract or pantethine was added to the basal medium [1]. The stimuli could, however, be compensated by addition of cysteine or other reducing agents to the medium. *

More detailed investigation showed later that both the homomeric disulfide of thiamine and several mixed disulfides of the allithiamine type are inactive as growth factors for *L. fermenti*, except when reduced or treated with cysteine [2-4]. It remained to establish whether a postulated thiamine pantetheine disulfide could account for the observed enhancement with pantethine. If so, this would require rather different physiological aspects for the thiamine pantetheine derivative when compared with the allithiamines.

Experimental. The mixed disulfide of the thiol form of thiamine and pantetheine (the mercaptoethanolamine of pantothenic acid) was first mentioned by Sahashi *et al.*[5] who observed a new spot on the chromatograms of a mixture of the two vitamins. It was presumed that the two compounds form a mixed disulfide:

$$\text{T}-\text{SS}-\text{T} + 2\,\text{LBF}-\text{SH} \rightleftharpoons 2\,\text{T}-\text{SS}-\text{LBF} \quad \ldots a$$

We prepared T−SS−LBF through incubation of equimolar amounts of the parent compounds (on sulfhydryl basis) in a 1/15 M phosphate buffer solution of pH 7.5−8.5 for 20−30 min at 37°C.

T−SS−T was prepared according to Zima *et al.*[6] After repeated recrystallization from aqueous acetone the product was tested bioautographically for purity as below.

Commercial pantethine (Nutritional Biochemicals Inc.) was reduced to LBF−SH by forcing the solution of 100 mg LBF−SS in 5 ml 0.5 N H_2SO_4 through a 0.8 × 12 cm column of freshly amalgamated 20 mesh zinc granules (Jones reductor) with the aid of nitrogen gas, according to the reduction of coenzyme-A by Buyske *et al.*[7]

In analogy with the work of Matsukawa and Yurugi [8] on the thiamine cysteine mixed disulfides we prepared T−SS−LBF also through

$$2\,\text{T}_{\text{ol}} + \text{LBF}-\text{SS} \rightleftharpoons 2\,\text{T}-\text{SS}-\text{LBF} \quad \ldots\ldots b$$

Chromatographic analysis and growth test gave similar results with both preparations according to equations *a* and *b*, in agreement with the presumed analogy.

The reaction mixtures were subjected to paper chromatographic separation in several solvent systems (*viz.*, *n*-butanol : water : acetic acid 125:125:30; *n*-butanol: ethanol : acetic acid:water 4:1:1:10; *iso*-butanol: pyridine : water 3:2:5; and *tert.*-butanol : water : methylethylketone 2:2:1). Ascending chromatograms were carefully dried in hot air current, and developed bioautographically by laying on the surface of large agar plates in which the basal medium was completed with 2 % agar and seeded with a heavy suspension of *L. fermenti*. The technique was otherwise similar to our agar cup plate method described elsewhere [9].

Separation of the T−SS−T reaction mixture resulted in more than two spots as shown in the corresponding columns of Fig. 1. This was also the case with the other solvent systems used. Among the spots the factor next to the fastest moving component was identified as T−SS−T by its R_F, and by its disappearance on the bioautograms which were developed with a basal medium lacking cysteine. Under these conditions all components other than T−SS−T were active including the fastest moving factor. Thus the fastest moving factor behaved differently as the starting materials used for the synthesis, or as the other predictable compounds of known structure (*e. g.*, T−SS−T) and must be considered to be the actual T−SS−LBF complex.

Meanwhile, the slowest moving spots of columns II and III in Fig. 1, are evidently representing the thiol form of thiamine since this appears only at slightly alkaline pH according to the equilibrium:

$$\text{T}_{\text{tz}} \rightleftharpoons (\text{T}_{\text{pseudobase}}) \rightleftharpoons \text{T}_{\text{ol}} \quad \ldots\ldots\ldots c$$

Considering eqn. *c* as well as eqns. *a* and *b*, the partition in column III reflects the simultaneous presence of all factors of the three equilibria.

* *Abbreviations:* T, T_{tz}, T_{ol} and T−SS−T denote thiamine, the thiazole (amino) form of thiamine, the thiol form of the same, and the homomeric disulfide form. For pantethine and pantetheine, respectively, the letters LBF−SS and LBF−SH were applied in view of the early name of these factors (Lactobacillus Bulgaricus Factor) while the mixed disulfide of thiamine with pantetheine is represented with T−SS−LBF.

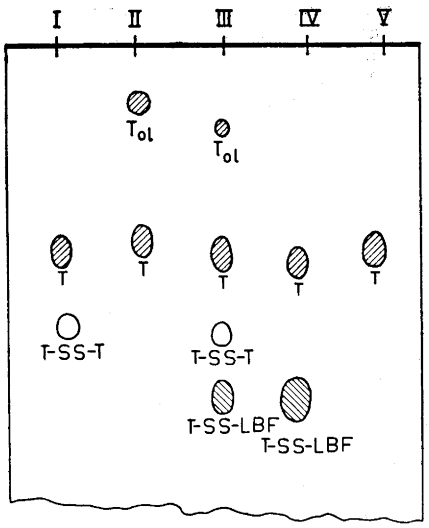

Fig. 1. Selective bioautography of thiamine pantethein disulfide (T—SS—LBF). Ascending chromatography on Whatman No. 1 paper in *n*-butanol : ethanol : acetic acid : water 4:1:1:10 solvent system (upper layer). Bioautographic development was made with *L. fermenti* 36 by using media with or without cysteine. Unshaded zones do not support growth in absence of cysteine.

Attention should be paid in this connection to the fact that in the agar plate bioautography, T—SS—LBF is active for *L. fermenti* both in presence and in absence of cysteine. This is also the case with the thiol form of thiamine, the activity of which could not be decided with the tube test [3].

The fastest moving spot of T—SS—LBF marked in column III of Fig. 1, was subjected to elution with ethanol water mixture and rechromatographed. Column IV of Fig. 1 shows that thiamine will be easily liberated from the eluate despite the mild conditions of the elution and that of the chromatographic procedure. Also slight acidity or addition of traces of cysteine will shunt reaction *b* to the left.

Batches of the eluate of the T—SS—LBF spot were subjected to quantitative test with the agar cup plate method and the tube method using *L. fermenti*. Analysis in absence and in presence of cysteine in the medium showed that T—SS—LBF i fully active as a growth factor (tube test in Fig. 2) in contrast to our experience with the homomeric and heteromeric disulfides of thiamine [4]. It is, however, not decided at present whether the thiamine like activity of thiamine pantetheine disulfide can be considered as a physiological peculiarity or should be attributed to the chemical lability of this compound.

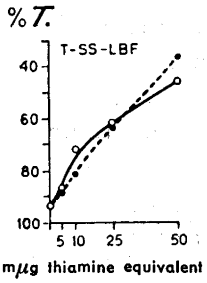

Fig. 2. Quantitative test of thiamine pantetheine disulfide with *L. fermenti* 36 on fluid media with 500 μg cysteine per ml and without cysteine (the latter marked with dotted line). Growth was measured turbidimetrically in dublicates at 5 950 Å and is expressed in per cent transmittance (% *T.*).

Conclusion. Thiamine pantetheine disulfide functions as a growth factor for *Lactobacillus fermenti* similarly to the thiazole and thiol forms of thiamine under experimental conditions which are unfavourable for the utilization of thiamine disulfide or allithiamine analogues.

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1. Bánhidi, Z. G. *Acta Chem. Scand.* **12** (1958) 517.
2. Bánhidi, Z. G. *Biochem. Z.* **332** (1959) 77.
3. Bánhidi, Z. G. *J. Bacteriol.* **79** (1960). *In press.*
4. Bánhidi, Z. G. *Acta Chem. Scand.* **13** (1959) 1901.
5. Sahashi, Y., Funahashi, S., Yamashita, K. and Akatsuka, T. *J. Biochem.* (*Tokyo*) **41** (1954) 463.
6. Zima, O., Göttmann, G., Hoffmann, A., Hepding, L., and Hotovy, R. E. *Merck's Jber. Pharm.* **67** (1953) 1.
7. Buyske, D. A., Handschumacher, R. E., Schilling, E. D. and Strong, F. M. *J. Am. Chem. Soc.* **76** (1954) 3575.
8. Matsukawa, T. and Yurugi, Sh. *Science* **118** (1953) 109.
9. Bánhidi, Z. G. *Analyst* **84** (1959).

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