# STUDIES ON THE AEROBIC MESOPHILIC BACTERIA WITH DISTICTLY BULGED SPORANGIUM

## I. NUTRITIONAL REQUIREMENTS OF *BACILLUS THIAMINOLYTICUS*

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Thiamin decomposing enzyme "Aneurinase" or "Thiaminase" has been found in certain shell-fishes[1], fishes[2], and plants[3]. Recently thiaminase producing bacteria were isolated in Japan and were named "*Bacillus thiaminolyticus*" and "*Bacillus aneurinolyticus*" (see first report of this investigation). The bacteriolgical properties of the first species were reported in the previous paper[4].

In 1952, Kimura et al. isolated several strains of anaerobic sporulating bacterium capable of producing thiaminase. This anaerobes were named "*Clostridium thiaminolyticum*".

*B. aneurinolyticus* grew readily in a synthetic medium containing glutamic acid as a sole source of nitrogen and glucose as a source of carbon. The metabolism of the bactaria has been studied by Kimura and Liao[5] and by Kishida[6].

In the present paper, the author studied the nutritional requirements of *B. thiamionlyticus* and found some chemically defined medium containing many kinds of amino acids to be as adequate as is the nutrient broth for the growth of this organism. This synthetic medium enabled the study of variable compounds which might influence the bacterial growth. *Bacillus alvei*, which was considered to be related to *B. thiaminolylicus* in the previous reports, was also studied when necessary.

## MATERIALS AND METHODS

Among the strains described in the preceding paper, *B. thiaminotyticus* strains no. 22, 34, 101, 254 and 362, *B. alvei* strains no. 662 and 668 were used for the study of amino acids and glucose requirements. *B. thiaminolyticus* strain 101 was investigated for the study of the compounds which might influence the bacterial growth.

Modified amino acids-glucose medium based on the formula reported by Johnson and Röttger[7] was used as a basal medium. Composition of the medium was listed in Table I. Amino acids mixture and salts solution were sterilized separately by autoclaving at 15 pounds for 10 minutes. After mixing both fluids, the readjustment of the mixture with N NaOH to give a pH of 7.2 was followed

by the aseptical addition of tryphan which had been sterilized by filtering through Seitz-filter. Two ml of the amino acids-salts mixture thus obtained was distributed in the 13 × 140 mm test tubes. Glucose which was sterilized by filtration and supplements were added there to and the content of each tube was diluted with distilled water to make a total volume of 3.9 ml.

TABLE I

*Composition of the medium*
*(Double Strength)*

| | |
|---|---|
| Glycine | 50 mg |
| L-Alanine | 60 mg |
| DL-Valine | 80 mg |
| L-Asparagine | 100 mg |
| L-Glutamic Acid | 100 mg |
| L-Tryptophan | 50 mg |
| L-Tyrosine | 50 mg |
| D-Arginine Hcl | 50 mg |
| D-Histidine Hcl | 50 mg |
| L-Proline | 60 mg |
| DL-Methionine | 50 mg |
| DL-Leucine | 100 mg |
| DL-Isoleucine | 100 mg |
| DL-Serine | 70 mg |
| DL-Phenylalanine | 100 mg |
| L-Cystine | 100 mg |
| L-Lysine | 50 mg |

| | |
|---|---|
| Glucose | 1.0 g |
| Salts Solution | 25.0 ml |
| $K_2HPO_4$ | 2.25 g |
| Distilled Water | 500.0 ml |

| Salts Solution | |
|---|---|
| $MgSO_4.7H_2O$ | 10.0 g |
| $FeSO_4.7H_2O$ | 6.5 g |
| $Mncl_2.4H_2O$ | 0.355 g |
| Dis. Water | 1,250.0 ml |

Final pH 7.2

2-Methyl-4-amino-5-hydroxymethyl-pyrimidine, 4-methyl-5-$\beta$-hydroxyethyl-thiazole and amino-thiazole were stocked as $10^{-2}$ mol solution after autoclaving at 15 pounds for 10 minutes. If $10^{-4}$ mol in the final concentration, for example, was desired, 0.4 ml of $10^{-3}$ mol solution, which was obtained by adding aseptically 9 volumes of sterile water to one volume of the stock $10^{-2}$ mol solution, was added to the amino acids-salts mixture under aseptical conditions.

For the sterilization of thiamin and its related compounds these were dissolved in a small volume of N Hcl directly, after 2 hours of incubation at room temperature, neutralized by calculated amounts of N NaOH, and were diluted by sterilized distilled water to make a concentration of $10^{-3}$ mol.

Through-out these studies, aqueous solution of the following chemicals were used as vitamin mixture; riboflavin 10$\gamma$, pyridoxine 100$\gamma$, nicotinic acid 100$\gamma$, para-aminobenzoic acid 10 , i-inositol 100$\gamma$, biotin 5$\gamma$, ca-pantothenate 50$\gamma$, folic acid 10$\gamma$, thymine 50$\gamma$, uracil 50$\gamma$, dissolved in 100 ml of distilled water. The solution of these chemicals was sterilized through Seitz-filter, and stored in a refrigerator. The volume of the mixture used was 0.2 ml in each tube.

Cells grown on nutrient agar slant for 48 hours at 35 C. were harvested and washed twice with distilled water and suspended in distilled water. Then the suspension was centrifuged at 1,000 r. p. m. for 10 minutes, and 0.1 ml of the resulting homogenous, barely turbid bacterial suspension was used for the inoculation. In 0.1 ml of the inoculum 50,000 to 100,000 viable cells were con-

tained, as determined by standard plating methods.

The growth was checked visually or determined by the use of Ito's photoelectric colorimeter with 560 mμ filter after suitable incubation at 35 C. In the later case, the cultures were shaken at intervals of three to four hours to prevent pellicle formation and to secure uniform turbidity.

## Experimental Results

I) Amino acids requirements: The essential amino acids were determined by leaving out one amino acid at a time from the basal medium. As seen from Table II, leucine, isoleucine, proline and asparagine were essential for all strains of *B. thiaminolyticus*, while two strains of *B. alvei* required isoleucine, tryptophan, valine and methionine.

Table II

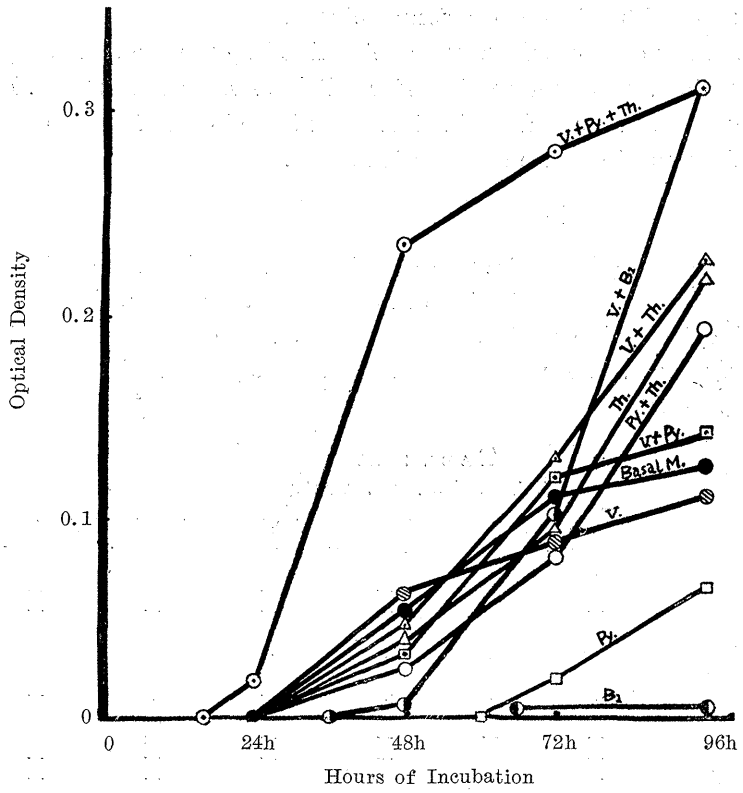
*Amino acids requirements*

| | Al. 662 | Al. 680 | An. 22 | An. 34 | An. 101 | An. 254 |
|---|---|---|---|---|---|---|
| Complete Medium | + | + | + | + | + | + |
| C. M. -Leucine | + | + | − | − | − | − |
| 〃 -Isoleucine | − | − | − | − | − | − |
| 〃 -Serine | + | + | + | ± | ± | + |
| 〃 -Phenylalanine | + | + | + | + | + | + |
| 〃 -Lysine | + | + | + | + | + | + |
| 〃 -Glycine | + | + | + | − | + | − |
| 〃 -Alanine | − | + | + | − | ± | |
| 〃 -Valine | − | − | − | − | ± | |
| 〃 -Asparagine | − | ± | − | − | − | − |
| 〃 -Glutamic Acid | − | ± | ± | ± | − | − |
| 〃 -Tyrosine | − | + | + | + | ± | |
| 〃 -Tryptophan | − | − | ± | + | ± | |
| 〃 -Arginine Hcl | − | + | + | + | | + |
| 〃 -Histidine Hcl | − | + | + | − | + | |
| 〃 -Proline | − | ± | − | − | − | − |
| 〃 -Methionine | − | − | ± | − | + | |
| 〃 -Glucose | − | + | − | + | + | − |

+ ; Growth, ± ; Questionable Growth, − ; No Growth

II) Glucose requirements: Optimum concentration of glucose for the growth of *B. thiaminolyticus* in the basal medium was 0.075 to 0.1 per cent when judged visually, some inhibition was recorded at 0.5 and complete inhibition at 1.0 per cent while *B. alvei* were able to grow in a medium containing 1.0 per cent glucose. When glucose was omitted from the basal medium, the growth of two strains of *B. thiaminolyticus* and one of *B. alvei* was recorded as negative, and that of the remaining two of the former species and one of latter species as slightly positive.

III) Effect of 2-methyl-4-amino-5-hydroxymethyl-pyrimidine, 4-methy-5-β-hydroxyethyl-thiazole, thiamin and vitamin mixture on the growth of *B. thiaminolyticus*: The effect upon strain no. 101 was summarized in Fig. I. Thiamin was far from stimulating the growth of *B. thiaminolyticus*, it inhibited the growth. The inhibitory effect of thiamin is neutralized by the vitamin mixture and




Py.: 2-methyl-4-amino-5-hydroxymethyl-pyrimidine
Th.: 4-methyl-5-$\beta$-hydroxyethyl-thiazole
V.: Vitamin mixture

Fig. 1 Effect of Thiamin, Thiazole, Pyrimidine and Vitamin mixture on *B. thiaminolyticus* 101 growth in synthetic medium.

the growth finally became abundant. Pyrimidyl moiety of thiamin inhibited the growth while the thiazole moiety has a favorable effect on the growth. When both pyrimidine and thiazole moieties of thiamin had been incorporated in the synthetic medium together with the vitamin mixture, the growth was more abundant than that in the medium containing the pyrimidine and the mixture, than the thiazole and the mixture, or than both the moieties and the no vitamin mixture. The vitamin mixture alone had no appreciable effects on the growth of the organism.

The author is now investigating the factor or factors in the vitamin mixture, which are responsible for neutralizing the inhibitory effect of thiamin and promoting the growth together with pyrimidine and thiazole moieties of thiamin, and some data has been collected which will be reported elsewhere.

In the earlier period of this investigation the author found the apparently

favorable effect of thiamin upon *B. alvei* at the concentration of $10^{-4}$mol (see discussion of the first report). Later, however, it was found that it was not always the case and so it is admitted that the study of the effect of thiamin and its components on the growth of *B. alvei* is far from complete and no justifiable conclusions can be drawn, except that these compounds have at least no unfavorable effects on the growth of *B. alvei* below the concentration of $2 \times 10^{-4}$ mol.

IV) The effect of thiamin analogues and "Amino-thiazole": The thiamin analogues studied were "Oxythiamin" (4-methyl-5-β-hydroxyethyl-N [(2'-methyl-4'-hydroxypyrimidyl-5') methyl]-thiazolium chloride), "Heteropyrithiamin" (N-(2-methyl-4-amino-pyrimidyl-5-methyl)-pyridimium bromide), and "Benzthiazole-thiamin"(N-[(2'-methyl-4'-amino-pyrimidyl-5')-methyl]-benzothiazolium bromide hydrobromide).

The "Oxythiamin" whose anti-thiamin activities were reported by Slobodin and Ziegel[8] and whose anti-thiaminase activities by Soodek and Cerecedo[9], were reported by Kishida[6] to inhibit the growth of *B. aneurinolyticus* at a concentration of $3 \times 10^{-4}$ mol, and by Kimura and Liao[5] not to influence it at that of $2 \times 10^{-5}$ mol. The "Heteropyrithiamin", which was reported by Fujita et al.[10] to be produced from thiamin and pyridine by reversible base exchange reaction catalized by the thiaminase of *B. thiaminolyticus*, shell-fish and fish, was found by Kishida[6] as having growth-promoting activities upon *B. aneurinolyticus*. The "Benzthiazole-thiamin" whose growth-inhibiting activities upon *Micrococcus pyogenes* was found by Kimura and Hayashi[11], was said to promote the growth of *B. aneurinolyticus*. The "Aminothiazole" was reported by Hayashi[12] to inhibit the growth of *Lactobacillus bifidus* at a concentration of $10^{-6}$ to $10^{-7}$ mol. As seen in Table III the "Heteropyrithiamin" stimulated the growth of *B. thiaminolyticus* at a concentration of $2 \times 10^{-4}$ mol. The "Oxythiamin" and the "Amino-thiazole", each at a concentration of $2 \times 10^{-4}$ mol, and the "Benzthiazole-thiamin" at a concentration of $10^{-4}$ mol inhibited the growth completely.

TABLE III

*Effect of oxythiamin, heteropyrithiamin, benzthiazolethidmin and amino-thiazole for the growth of B. thiaminolyticus in the synthetic medium*

| Supplements | Growth of *B. thiaminolyticus* 101. Light Transmission per cent | |
|---|---|---|
| | 48h. | 96h. |
| Oxythimin | 100 | 100 |
| Heteropyrithiamin | 80 | 80 |
| Benzthiazole-thiamin | 100 | 100 |
| Amino-thiazole | 100 | 100 |
| Non (Basal Medium) | 100 | 80 |

Ito's colorimeter, 560 mμ filter, uninoculated medium 100. Final concentration of the supplements, $2 \times 10^{-4}$ M. each, except for Benzthiazole-thiamin, concentration $10^{-4}$ M.

V) Extra- and intracellular thiamin content: The author found that by *B. thiaminolyticus* in a liquid medium an unkown substance is produced, which when shaken with concentrated sodium hydroxide converts to a substance having thiochrome like fluorescence. This substance, which is referred to here as X-substance, is absorbed in zeorite as is thiamin and can be eluted from it by 25 per cent potassium chloride or acetic acid water of pH 4.0. Thiamin is insoluble in butanol but becomes soluble after having been converted to thiochrome. On the other hand, this X-substance is easily transferred to butanol both before and after being converted to the fluorescent substance, whose fluorescence decreses remarkably after standing in a dark room overnight. The X-substance is absorbed by active carbon and is eluted from it by acetic acid-ethanol but not by neutral ethanol or acetic water. It is soluble in alcohol and acetone but not in benzole and stable below pH 9.5 and withstands boiling more than one hour.

For the purpose of assaying thiamin and the X-substance, the cultures were centrifuged and separated into the supernate and the bacteria, the former, after treatment with Takadiastase-solution, was abosorbed into the zeorite beds, which was prelminary treated as described by Hennessy[13], the latter, after being washed once with water, was suspended in a small volum of acetic acid water of pH 4.5. The bacterial suspension was heated at 80 C. for 20 minutes to extract intracellular thiamin and X-substance, and then centrifuged. Aliquot volume of the supernate obtined was treated with Takadiastase solution and was allowed to pass through another zeorite bed. After being washed, through the two zeorite beds, one of which absorbed the intra- and another the extra-celluar thiamin and X-substance, a boiling solution of 25 per cent potassium chloride was passed to elute thiamin and the X-substance. The zeorite elute was shaken with butanol and centrifuged. The butanol layer to which the X-substance had been transferred was distributed to a test tube, which served as a blank and to a centrifuging tube. The butanol solution in the centrifuging tube was shaken with 40 per cent sodium

TABLE IV

*Thiamin and X-substance in the culture of B. thiaminolyticus 101 after 36 and 96 hours' incubation at 35 C in nutrient broth*

| Thiamin and X-substance* (γ per 100 ml) | | None | | Supplements ($10^{-4}$mol) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Py. and Th. | | Thiamin | |
| | | Thiamin | X-sub. | Thiamin | X-sub. | Thiamin | X-sub. |
| 36-hr. | Supernate | — | 3 | — | 4 | 300 | 3 |
| | Cells | 0.12 | — | 0.12 | — | 1.1 | — |
| 96-hr. | Supernate | — | 50 | — | 160 | 250 | 120 |
| | Cells | 0.24 | — | 0.3 | — | 3.2 | — |

* As X-substance was titrated by standard thiochrome solution, its figures indicate merely the relative amount per 100 ml of medium.

hydroxide and centrifuged. An aliquot volume of the butanol in the centrifuging tube was used for the fluometric determination, using standard thiochrome solution as in the case of assaying thiamin. Thus figures of X-substance presented in Table IV merely indicate the relative amount of the substance in the cultures.

Following the preliminary butanol extration, the zeorite elute was used for the determination of thiamin with the thiochrome method by means of cyanogen bromide[14)15)16)]. As seen from Table IV the X-substance could be detected only extracellularly and thiamin only intracellularly.

## Discussion and Summary

*B. thiaminolyticus* and *B. alvei* grow well in a synthetic medium containing 17 amino acids, glucoce and salts.

Among strains tested it could be observed that there were different amino acids requirement depending on the variety of strains belonging to the same species, but that the indispensable amino acids for all strains of *B. thiaminolyicus* tested were leucine, isoleucine, proline and asparagine and for those of *B. alvei* isoleucine, valine, tryptophan and methionine.

A small amount of glucose stimulated the growth of both species in synthetic medium, but 0.5 to 1.0 per cent of the sugar inhibited the growth of *B. thiaminolyticus* but not that of *B. alvei*.

Thiamin alone inhibits the growth of *B. thiaminolyticus*, but in the presence of vitamin mixture the growth may be initiated late and finally reaches to a level comparable to that of growth in the medium which contains pyrimidine and thiazole moieties of thiamin and vitamin mixture. This medium permits the best growth to *B. thiaminolyticus* of any media tested. These observations indicate that thiamin is inhibitory for the bacterial growth and that after initiation of growth, thiamin breaksdown and the resulting products promote growth. The inhibitory effect of "Oxythiamin", may be due to the inability of the thiaminase to attack this anti-thiamin. The inhibitory effect of amino-thiazole indicates that thiazole plays an important role in the metabolism of *B. thiaminolyticus*.

"Heteropyrithiamin" was found by Fujita et al.[10)] to be produced from thiamin and pyridine by the enzymatic activity of thiaminase, and pyrimidyl compounds analogous with the "Heteropyrithiamin" were considered by them to be produced from thiamin in natural conditions without pyridine. The fact that the "Heteropyrithiamin" promotes the growth of the organisms, suggests that the enzyme products in natural conditions may have favorable effects on the growth of *B. thiaminolyticus*.

The author demonstrated that an unknown substance by means of shaking with concentrated sodium hydroxide, was produced by *B. thiaminolyticus*. Therefore, thiamin in the cultures of the *B. thiaminolyticus* must be assayed after this

misleading substance has been removed by preliminary butanol extraction.

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