Thiamine Sparing Action of Ascorbic Acid on *Lactobacillus Fermenti* 36.* (20195)

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The stimulatory effect of ascorbic acid on lactic acid bacteria has recently been demonstrated. Kitay *et al.*(1) found that ascorbic acid was rarely active and only at higher concentrations. It promoted growth of *L. leichmanii* 313 and *L. acidophilus* 4913 but was inactive in promoting growth of *L. acidophilus* 832, *L. delbrueckii* 730, and *Leuconostoc citrovorum* in defined media. In the study on the growth of *L. casei*, Rickes *et al*(2) concluded that the effectiveness of ascorbic acid and d-isoascorbic acid in further increasing the rate of growth of this microorganism presumably was due to the reducing effect. Katznelson(3) found that sodium thiosulfate and ascorbic acid may partially replace the thiamine requirement of *Bacillus paraalvei*. Recently, using *L. fermenti* 36 in the microbiological assay of thiamine(4), we have noted that when as little as 0.4 mg of ascorbic acid was added to 10 ml of assay medium, the growth effect of thiamine was greatly enhanced. Furthermore, the organism responded quantitatively to ascorbic acid in a thiamine-free medium, reaching a maximum at 1 mg per tube. Since thiamine is intimately concerned with carbohydrate metabolism, especially with the degradation of pyruvic acid, the observation of the thiamine sparing action of ascorbic acid on growth of this microorganism throws interesting light on the role of ascorbic acid.

It is the purpose of this paper to attempt to clarify in part the interrelationship of these two vitamins.

Methods. *Lactobacillus fermenti* 36 was grown in the medium used for thiamine assay (5,6). Varying amounts of thiamine or ascorbic acid and other substances to be tested were measured into tubes, diluted to 5 ml, and 5 ml of medium added. Boiled alkali-digested peptone was used in the medium in order to secure a lower blank. The tubes were plugged, steamed and inoculated with a very dilute suspension of *L. fermenti* 36 inoculum. Growth was measured turbidimetrically after 16 to 24 hours of incubation with a Klett-Summerson photoelectric colorimeter, using a 54 filter. The results are expressed as Klett units.

Results. Stimulatory effect of ascorbic acid on growth of L. fermenti 36 supplied with suboptimum amounts of thiamine. Table I shows that the growth effect of thiamine was greatly enhanced when 2 mg or 5 mg of ascorbic acid were added either before steaming or aseptically after steaming. During the early growth period, thiamine will not prevent the stimulatory effect of ascorbic acid nor will ascorbic acid prevent the similar effect of thiamine.

Effect of thiamine and ascorbic acid on growth of L. fermenti 36. When graded amounts of ascorbic acid were added, either before steaming or aseptically to the thiamine-free medium, the early growth of *L. fermenti* 36 responded quantitatively. A typical set of data is shown in Table II. However, when the incubation period was prolonged to 72 hours thiamine promoted better growth and more acid production than did ascorbic acid. This indicates that ascorbic acid cannot completely replace thiamine in the growth of this microorganism. It suggests that ascorbic acid may permit the synthesis of thiamine to a limited degree or that ascorbic acid may partially perform the physiological role usually carried out by thiamine. When pyrithiamine was added to the medium this inhibitor was much more effective against ascorbic acid than against thiamine. Furthermore, much more pyrithiamine was required to have the same inhibitory effect when thiamine and ascorbic acid were present together than when they

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TABLE I. Stimulatory Effect of Ascorbic Acid on Growth of *Lactobacillus fermenti* 36 when Supplied with Suboptimum Amounts of Thiamine.

Thiamine level, γ	Turbidity reading: Ascorbic acid added before autoclaving: 0	5 mg/tube	2 mg/tube	Ascorbic acid added aseptically: 5 mg/tube	2 mg/tube
.00	27	67	69	53	58
.01	41	93	99	84	82
.02	48	102	111	99	100
.03	52	116	122	97	110
.05	55	132	133	114	113

Turbidities were measured with a Klett-Summerson photoelectric colorimeter, using a 54 filter, after 17 hr incubation at 37°C and expressed as "Klett Units." Assay medium described in (4) was used.

TABLE II. Effects of Thiamine and Ascorbic Acid on Growth of *Lactobacillus fermenti* 36.

Thiamine, γ (only)	Turbidity reading	Ascorbic acid, mg (only)	Turbidity reading: Added before autoclaving	Added aseptically
.00	27	.0	26	27
.01	41	.5	47	51
.02	48	1.0	62	54
.03	52	2.0	70	54
.05	55	5.0	68	52

Turbidities measured with a Klett-Summerson photoelectric colorimeter, using a 54 filter, after 17 hr incubation at 37°C and expressed as "Klett Units." Assay medium described in (4) was used.

were used separately. These data as recorded in Table III suggest the synergistic effect of ascorbic asid on thiamine activity.

In studying the mechanism of the growth-promoting effect of ascorbic acid on *L. leichmanii*, Welch *et al.*(7) suggested that the apparent microbiological activity of ascorbic acid may be attributed to the conversion of oxidation products of vit. B_{12} to microbiologically more active forms. It seems unlikely that a similar role for ascorbic acid would apply in this case, because if the assay medium contains some oxidized form of thiamine, then addition of ascorbic acid, which would convert the oxidized product back into thiamine, should not further enhance the growth-promoting activity of thiamine as the latter was present in sufficient quantity in the assay medium. Stokstad *et al.*(8) reported that thioglycolic acid and ascorbic acid protected vit. B_{12} against destruction during autoclaving of assay media containing the vitamin. Our results show that when the ascorbic acid was added aseptically (sterilized by filtration) to the medium after steaming it also enhanced the growth-promoting activity of thiamine. Therefore, this would seem to indicate that ascorbic acid must have a special function on the carbohydrate metabolism. In a separate experiment, either thioglycolic acid or

TABLE III. Inhibition Effect of Pyrithiamine on Utilization of Thiamine or Ascorbic Acid for Growth of *L. fermenti* 36.

Pyrithiamine added/tube, γ	Turbidity reading: Thiamine level, γ: 0	0.05	0.1	Pyrithiamine added/tube, γ	Turbidity reading: Ascorbic acid level, mg: 0.5	1.0	1.5	Pyrithiamine added/tube, γ	Turbidity reading: 1 mg vit. C + 0.05 γ B_1	1 mg vit. C + 0.1 γ B_1
0	19	96	116	0	47	54	55	0	114	133
.1	5	89	—	.01	5	6	38	.1	116	—
.2		68	—	.02	5	8	7	.2	97	—
.3		45	93	.03	5	5	6	.3	74	133
.4		25	72	.04	5	5	5	.4	54	132
.5		—	36					.5	—	109
.6			15					.6	—	89
Amt pyrithiamine required for 50% growth inhibition		.29	.44		<.01	<.01	.014		.38	.71

Incubation period 16 hr.

TABLE IV. Effect of Several Enzyme Inhibitors on Utilization of Thiamine or Ascorbic Acid for Growth of *L. fermenti* 36.

Substances added	Range used/tube	Thiamine level		Ascorbic acid level	
		1.0 γ	0.5 γ	2.0 mg	1.0 mg
	mg	mg	mg	mg	mg
Iodoacetate	0– .4	.16*	.16	.40	.38
2,4-dinitro-phenol	0–2	.16		†	
KCN	0–2	†		†	
NaF	0–2	2.0		†	
Malonic acid	0–2	†		†	

Incubation period 16 hr.
* Amount of inhibitor necessary to cause 50% inhibition.
† No inhibition.

cysteine in an amount of 2 mg per 10 ml medium had shown to enhance the growth-promoting activity of thiamine by approximately 15%. However, these two compounds do not support growth of *L. fermenti* 36 without the presence of thiamine. This result further proves that the thiamine sparing action of ascorbic acid is not due to its reducing effect. When sodium ascorbate was added to the assay medium containing no glucose, no growth of this organism was observed even in the presence of thiamine indicating that ascorbic acid could not be used as substrate.

Effect of several metabolic inhibitors on Growth of L. fermenti 36 in presence of thiamine or ascorbic acid. In order to determine whether the thiamine and ascorbic acid have the same mechanism on the growth-promoting effect, several other known metabolic inhibitors were added individually to assay media which contained either thiamine or ascorbic acid. Increasing amounts (0-2 mg) of the inhibitors were added to 10 ml of the assay media. The growth of *L. fermenti* 36 was measured after 16 hours of incubation. The 50% inhibition indices of all metabolic inhibitors used were determined and are recorded in Table IV. Iodoacetate, in a concentration of 0.16 mg per 10 ml assay medium, was found to cause 50% inhibition of the growth promoted by either 1 γ or 0.5 γ of thiamine. Higher concentrations, 0.40 mg and 0.38 mg, were required respectively in the cases of ascorbic acid. With 2,4-dinitrophenol, 0.16 mg was required to inhibit 50% of the growth which was promoted by 1 γ of thiamine and no inhibition was observed when ascorbic acid was present. A difference was also noted between the growth-promoting effect of thiamine and ascorbic acid when sodium fluoride was used. These results all pointed to the fact that ascorbic acid must function in a different metabolic pathway when compared to thiamine. 2,4-dinitrophenol has been shown to retard pyruvic acid oxidation by brain tissue(9). Addition of fluoride presumably inhibited the breakdown of phosphoglyceric acid. In the growth of *L. fermenti* 36 which was promoted by ascorbic acid in the absence of thiamine no inhibition by fluoride was observed. This would indicate that oxidation of glucose by *L. fermenti* 36 may by-pass the step inhibited by fluoride when ascorbic acid is present.

Thiamine Synthesis of L. fermenti 36 Grown in Assay Medium Containing Thiamine, Ascorbic Acid or both. To one liter of assay medium(5), 10 γ of thiamine or 200 mg ascorbic acid were added; the medium was then sterilized by two 30-minute periods of steaming. Ten drops of concentrated inoculum of *L. fermenti* 36 were used and the flasks were incubated at 37°C for 18 hours. The cells were centrifuged, washed twice with distilled water, and finally dispersed in water and diluted to 50 ml. One-half (25 ml) of of this cell suspension was digested with a takadiastase and papain mixture to liberate the thiamine(5). Thiamine determination was performed microbiologically using *L. fermenti* 36 as a test organism. A modified(4,5) assay medium which contained an additional 20 mg maltose and 2 mg ascorbic acid per 10 ml assay tube was used. The other half of the cell suspension was dried in order to determine the amount of cell production.

The experiment was repeated 3 more times. In the second experiment the growth was found to be very light after 18 hours, so growth was allowed to continue for 44 hours. In the third and fourth experiments both thiamine and ascorbic acid were included in the assay medium. In the fourth experiment, the thiamine content of the cells was determined by the thiochrome method(10), as a further check that thiamine was the factor being measured. The results are shown in

TABLE V. Thiamine Synthesis of *L. fermenti* 36 Grown in Thiamine Assay Medium Containing Either Thiamine, Ascorbic Acid or Both.

	Substance added in thiamine assay medium					
	Thiamine, 10 γ/liter		Thiamine, 10 γ and ascorbic acid, 200 mg/liter		Ascorbic acid, 200 mg/liter	
Incubation, hr	Cells production, g/liter	B_1 content, γ/g	Cells production, g/liter	B_1 content, γ/g	Cells production, g/liter	B_1 content, γ/g
18	.580	12.8			.159	6.6
44	.345	9.6			.148	4.2
24	.720	11.8	1.020	13.5	.356	7.0
24*	.250	6.4	.366	6.9	.086	3.9

* *L. fermenti* 36 ATCC 9338 used in this experiment. Thiamine values determined by thiochrome method. Fluorescence measured with a Beckman DU spectrophotometer.

Table V. The thiamine content of *L. fermenti* 36 cells grown in a medium containing ascorbic acid was found to be about one-half of the amount present in the cells grown in the thiamine medium. Furthermore, when both thiamine and ascorbic acid were present in the assay medium, not only was more growth of *L. fermenti* 36 observed, but the amount of thiamine in the cell was found to be increased by 10 to 15% over the cells grown in the thiamine medium. From this result, it may be assumed that the stimulatory effect of ascorbic acid on growth of *L. fermenti* 36 may function in part by stimulating the synthesis of thiamine.

Summary. 1. Ascorbic acid has been found to have thiamine sparing action on the growth of *L. fermenti* 36. The addition of both ascorbic acid and thiamine gives a greater growth response than either one when added alone. 2. Addition of pyrithiamine inhibits the utilization of ascorbic acid for growth of *L. fermenti* 36 in a thiamine-free medium. This result shows that ascorbic acid may function in part by stimulating the synthesis of thiamine. 3. Addition of iodoacetate, 2,4-dinitrophenol or sodium fluoride to the thiamine assay medium containing either thiamine or ascorbic acid shows the difference in inhibition of growth of *L. fermenti* 36. These results indicate that ascorbic acid must also function in a different metabolic pathway as compared to thiamine in carbohydrate metabolism. 4. It was found that *L. fermenti* 36 was able to synthesize thiamine when ascorbic acid was present. With the presence of both ascorbic acid and thiamine in the assay medium, the cell was found to contain more thiamine than the cell grown in thiamine medium alone.

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