Studies on the Degradation and the Synthesis of Thiamine Phosphates. (III)

Mechanism of Enzymatic Synthesis of Thiamine Diphosphate (Cocarboxylase)

Ryokuero SUZUE*

(Suzuki Laboratory)

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In the previous reports[1)2)] the author has presented that cocarboxylase (CoC) was synthesized from thiamine in the presence of acetyl-phosphate (acetyl-P) by the enzyme of *Lactobacillus delbrückii*. In this paper the mechanisms of the enzymatic synthesis, especially concerning the meaning of acetyl-P and ATP would be discussed.

Lipmann[3)] discribed that ATP was synthesized from AMP and acetyl-P by *Lact. delb.*[3)] and several investigators reported that cocarboxylase was synthesized from V B_1 and ATP by yeast or liver, pyrophosphate bond being transferred from ATP to V B_1[4~7)]. Presumable presence of co-enzyme system in *Lact. delb.* preparation was also here studied.

EXPERIMENTAL AND ESTIMATION METHODS

Inorganic phosphorus was estimated colorimetrically by Allen's method[8)]. When an enzyme mixture was turbid, isobutanol extraction of the colored substance was employed as described in the second report[2)].

For the recognition of cocarboxylase the evolution of CO_2 from pyruvate was determined by Warburg's manometric method. ATP was prepared from rabbit muscle in this laboratory[9)]. The purity of ATP and AMP (Ishizu Seiyaku K. K.) was examined by PPC, which was developed with the solvent of methanol, 80% formic acid and water (80 : 15 : 5) for 6-6.5 hours and dried by a fan at room temperature. After the paper was sprayed with the Hanes-Isherwood's reagent[10)] (5 ml. of 60% perchloric acid, 25 ml. of 4% ammonium molybdate, 10 ml. of N-HCl and 60 ml. of H_2O) at the rate of 1 to 2 ml. per 100 square cm., inorganic P appeared as a yellow spot. The paper was dried at 85°C for 1 min. and exposed to ultra violet radiation at a distance of about 30 cm. from a germicidal lamp for 10 min. All of organic phosphate compounds appeared as blue spots[11)]. The *Rf* values of ATP and AMP were 0.01 and 0.39, respectively. From these results the preparation of ATP or AMP was ascertained not to contain any other phosphorous compounds.

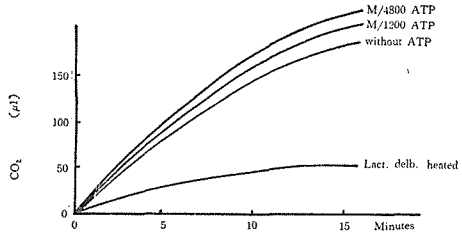
The amount of acetaldehyde in the incubation mixture was estimated as follows. To 1 ml. of test solution were added 7 ml. of water, 1 ml. of 10% Na-tungstate and 1 ml. of 2/3N H_2SO_4, and the mixture was centrifuged. The precipitate was discarded, and 8 ml. of the filtrate were distilled into 2 ml. of 2%

* 鈴 江 緑 衣 郎

$NaHSO_3$ until 5 ml. of distillate were obtained. One ml. of the distillate was mixed with 0.05 ml. of 5 % $CuSO_4$ and 8 ml. of conc. H_2SO_4. The mixture was colored by the addition of 0.2 ml. of 1.5 % *p*-hydroxybiphenyl solution in 0.5 % NaOH and the amount of aldehyde was determined colorimetrically, comparing to the standard paraaldehyde solution of 2μg per ml.[12].

RESULTS

1. Effect of ATP. Acetone powder of *Lact. delb.* cultivated in 100 ml. of malt solution for 48 hours, was suspended in 1.5 ml. of acetate buffer of pH 6.5, to which were added 50/3 mg. of acetyl-P, 5/3 mg. of V B_1 and $MgCl_2$ in M/300 of final concentration. The mixture was incubated for 90 min. at 37°C and the behavior of CO_2 evolution from pyruvate in the presence of the yeast apocarboxylase was observed (See the second report). As shown in Fig. 1, when the



Evolution of CO_2 from pyruvate

Fig. 1. Effect of ATP on the formation of CoC from V B_1.

concentration of ATP was low (M/4800), the evolution of CO_2 increased.

Velluz described that the amounts of acetaldehyde were proportional to the metabolism of pyruvate by yeast, as acetoin was hardly formed[18]. The results have demonstrated that when a small amount of ATP (M/4800) was added to the enzyme mixture, the production of acetaldehyde was increased (Fig. 2). It seems likely that a low concentration of ATP seems to stimulate the formation of CoC.

2. Effect of AMP. Effect of AMP was examined in the same enzyme system as in the former experiment. Addition of AMP also seemed to stimulate the evolution of CO_2 from pyru-

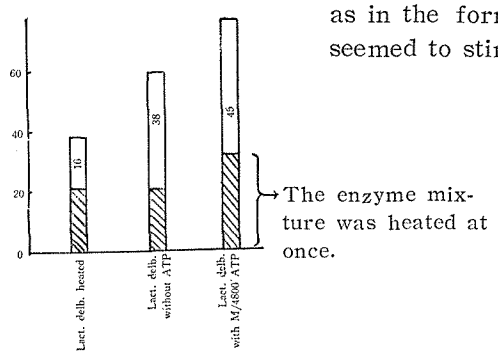
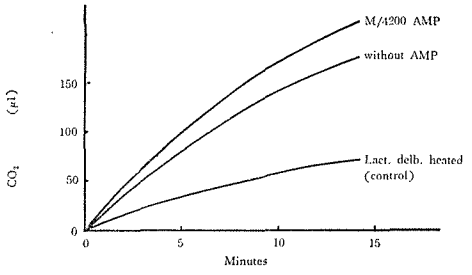


Fig. 2. Formation of acetaldehyde in the test mixture of pyruvate with CoC+ yeast apocarboxylase (μg).



Evolution of CO_2 from pyruvate

Fig. 3. Effect of AMP on the formation of CoC from V B_1.

Table 1. Effect of AMP on the formation of Δ7P in the incubation with V B_1 and acetyl-P by *Lact. delb.*

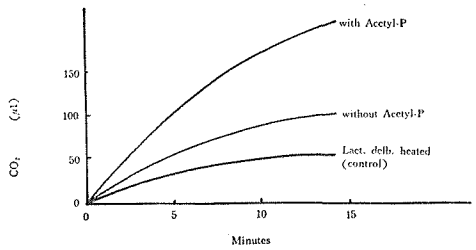
1/30 mg. AMP+16.6 mg. acetyl-P+*Lact. delb.*+$MgCl_2$ in 1.5 ml. of acetate buffer pH 6.5

	Amount of P in 0.05 ml. of the test mixture							
	With AMP				Without AMP (control)			
	N-HCl was added at once		Incubated at 37°C for 90 min.		N-HCl was added at once		Incubated at 37°C for 90 min.	
	Heated for 7 min. in N-HCl	Not heated	Heated for 7 min. in N-HCl	Not heated	Heated for 7 min. in N-HCl	Not heated	Heated for 7 min. in N-HCl	Not heated
Total amount of P(μg)	94	87	94	79	94	89	92	82
Δ7P(μg)	7		15		5		10	

vate in the test mixture (Fig. 3). It has been observed that a small amount of Δ7P has increased in the same enzyme mixture, as shown in Table 1.

On the other hand, if acetyl-P was used in these enzyme mixtures with AMP, the increase of CO_2 evolution was remarkable, while the evolution of CO_2 was a little, if acetyl-P was not added (Fig. 4).

These results might suggest that the phosphate bond of acetyl-P migh be transferred first to AMP to increase ATP in the incubation mixture. From Table 2 it might be supposed that when the concentration of AMP was low in the enzymatic mixture of acetyl-P, Lact. delb. and Mg^{++}, the phosphate bond of



Evolution of CO_2 from pyruvate

Fig. 4. Effect of acetyl-P on the formation of CoC from V B_1 in the presence of AMP.

Table 2. Effect of acetyl-P on the formation of Δ7P in the incubation with AMP by *Lact. delb.*

1/30 mg. AMP+16 mg. acetyl-P+*Lact. delb.*+$MgCl_2$ in 1.5 ml. of acetate buffer pH 6.5.

	The amount of P in 0.1 ml. of the test mixture			
	N-HCl was added at once		Incubation 37°C for 90 min.	
	Heated for 7 min. in N-HCl	Not heated	Heated for 7 min. in N-HCl	Not heated
Total amount of P(μg)	170	154	164	142
Δ7P(μg)	16		22	

Table 3. Effect of acetyl-P on the formation of Δ7P in the incubation with AMP by *Lact. delb.*
1 mg. AMP+16.6 mg. acetyl-P+*Lact. delb.*+$MgCl_2$ in 1.5 ml. of acetate buffer pH 6.5

	The amount of P in 0.1 ml. of the test mixture N-HCl was added at once		Incubation 37°C for 90 min.	
	Heated for 7 min. in N-HCl	Not heated	Heated for 7 min. in N-HCl	Not heated
Total amount of P(μg)	177	157	168	137
Δ7P(μg)	20		31	

acetyl-P might be transferred to AMP, and then the formation of ATP has resulted, while the concentration of AMP was high (thirty times as much as in the former), the formation of ATP appeared to occur comparatively to a low extent (Table 3). In the latter case it might be explained by the formation of ADP mainly occurred in a higher concentration of AMP.

3. Dialyzed *Lact. delb.* When dialyzed *Lact. delb.* was used as a enzyme, the evolution of CO_2 was not recognized (2). When to the mixture of AMR, Mg^{++}, acetyl-P and V B_1 was added the dialyzed *Lact. delb.* as apoenzyme, the evolution of CO_2 increased a little as shown in Fig. 5, while as already remarked the original acetone powder of *Lact. delb.* was capable to result evolution of

Table 4. Formation of Δ7P in the incubation with dialyzed *Lact. delb.*, V B_1 and acetyl-P.

	The amount of P in 0.1 ml. of the test mixture N-HCl was added at once		Incubation 37°C for 90 min.	
	Heated for 7 min. in N-HCl	Not heated	Heated for 7 min. in N-HCl	Not heated
Total amount of P(μg)	153	142	154	148
Δ7P(μg)	11		6	

Table 5. Formation of Δ7P in the incubation with dialyzed *Lact. delb.* and with dialysed *Lact. delb.*+boiled *Lact. delb.* concerning the formation of CoC from V B_1 in the presence of acetyl-P.

	Amount of P in 0.05 ml. of the test mixture Dialyzed *Lact. delb.*				Dialyzed *Lact. delb.* with boiled *Lact. delb.*			
	N-HCl was added at once		Incubated 37°C for 90 min.		N-HCl was added at once		Incubated 37°C for 90 min.	
	Heated for 7 min. in N-HCl	Not heated	Heated for 7 min. in N-HCl	Not heated	Heated for 7 min. in N-HCl	Not heated	Heated for 7 min. in N-HCl	Not heated
Total amount of P(μg)	69	68	74	73	89	84	88	65
Δ7P(μg)	1		1		5		23	

a great deal of Co_2. In the incubation mixture of dialyzed *Lact. delb.*, acetyl-P and V B_1, any increase of Δ7P was not observed (Table 4). However, if the boiled liquid of *Lact. delb.* was added to this incubation mixture with inactive dialyzed *Lact. delb.*, increase of Δ7P was remarkable (Table 5). This result was ascertained by the determination of CO_2 by Warburg's manometer.

When AMP was added to the mixture of dialyzed *Lact. delb.*, V B_1 and acetyl-P, increase of Δ7P was also demonstrated (Table 6). So it seemed likely that activator of this enzyme system which transferred the pyrophosphate bond to V B_1 was lost by dialysis. It will be described about this activating factor in the next section.

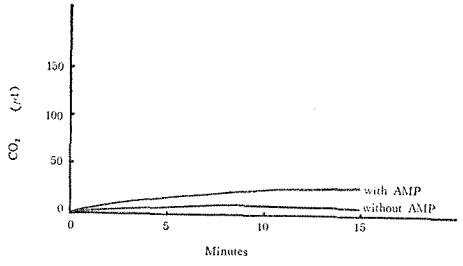
Table 6. Formation of Δ7P in the incubation with the dialyzed *Lact. delb.*, V B_1, acetyl-P and AMP.

	Amount of P in 0.1 ml. of the test mixture. N-HCl was added at once		Incubation 37°C for 90 min.	
	Heated for 7 min. in N-HCl	Not heated	Heated for 7 min. in N-HCl	Not heated
Total amount of P(μg)	161	159	165	153
Δ7P(μg)	2		12	

DISCUSSION

The experiment, in which acetyl-P was used as a phosphate donor, was described by Green and Meyerhof in 1952[14]. They used the phosphatase of the intestine and semen, and examined the transphosphorylation to glycerol, glucose, fructose, ribose and trehalose without the intermediate of nucleotide. As to the ability of transphosphorylation, acetyl-P was the first, and phosphocreatine was the second, and *p*-nitrophenol phosphate as well as phosphopyruvate had a weaker action, while ATP showed hardly such an activity.

The direct transferring of phosphate bond from acetyl-P to thiamine might be thought also to be possible. However, since AMP and ATP in its low concentration had some such effect on the formation of CoC in the enzyme system with *Lact. delb.*, so it seems likely that there might exsist at least two enzyme systems with *Lact. delb.* The former might be the system: acetyl-P+AMP→ ADP, or acetyl-P+ADP→ATP. The latter should be the system ATP+thiamine



Evolution of CO_2 from pyruvate

Fig. 5. Effect of AMP on the formation of CoC from V B_1 by dialyzed *Lact. delb.*

→CoC+AMP.

When a suspension of *Lact. delb.* was dialyzed, the activity of the enzyme which transferred pyrophosphate bond to thiamine in the presence of acetyl-P was lost. It was recovered by addition of boiled liquid of *Lact. delb.*, and this leads to raise a problem of some activating factors in the fluid.

SUMMARY

1. Transferring of phosphate to thiamine in the presence of acetyl-P was done by *Lact. delb.* In this case, a high concentration of ATP appeared to inhibit this action, but a low concentration of ATP stimulated this action. AMP had also effect of stimulating this action.

2. By dialysis the activity of this enzyme system of *Lact. delb.* was lost completely. The activity was recovered by addition of the boiled liquid of *Lact. delb.* and AMP+Mg^{++} reactivated the dialyzed enzyme only in a weak degree. The course of energy-rich phosphate compound formation was not affected by dialysis, but the enzymatic course of CoC formation was inactivated by dialysis.

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