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PHOSPHOTRANSACETYLASE OF *ESCHERICHIA COLI* B, ACTIVATION BY PYRUVATE AND INHIBITION BY NADH AND CERTAIN NUCLEOTIDES*

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SUMMARY

1. Phosphotransacetylase of *Escherichia coli* B has been found to be an allosteric enzyme which was activated by pyruvate and inhibited mainly by NADH. NAD^+ did not affect the enzyme activity.

2. ADP and ATP also inhibited the enzyme. But their inhibitory strengths were only one-quater and one-tenth of NADH, respectively. All these inhibitors affected the enzyme reaction, noncompetitively with CoA and sigmoidally with respect to the acetyl phosphate concentration. Pyruvate activated the enzyme by lowering K_m for acetyl phosphate without a change in v_{max}. A stimulatory site and an active site were distinct from each other.

3. Pyruvate repressed the inhibitory action of inhibitor and quite effectively activated the enzyme which had been inhibited by inhibitors, while it did not overcome the sigmoidicity of the reaction, indicating that inhibition and activation were based on separate mechanisms.

4. These inhibitors and activator were not consumed during the phosphotransacetylase reaction of *E. coli* B, in accordance with the result that phosphotransacetylase from *Clostridium kluyveri* was not affected by these effectors.

INTRODUCTION

The preceding report[1] described the purification of phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8) from *Escherichia coli* B. This enzyme had been found to be strongly inhibited by ATP[2] in the CoA-determining system with use of the partially purified enzyme of *E. coli* B (ref. 3). The nature of the inhibition by ATP, as well as the effects of other substances, have been further investigated with the highly purified enzyme, and it was found that the enzyme of *E. coli* B was an allosteric enzyme which was activated by pyruvate and inhibited mainly by NADH. This report deals with kinetic studies on the inhibition and activation of this enzyme. A part of this work was briefly communicated in a previous paper[4].

Abbreviations: acetyl-*P*, acetyl phosphate; PCMB, *p*-chloromercuribenzoate.

* This report represents Part XXII of *Investigations on Pantothenic Acid and its Related Compounds–Biochemical Studies (13)*; for Part XXI and *Biochemical Studies (12)*, see ref. 1.

MATERIALS AND METHODS

Phosphotransacetylase. The enzyme from *E. coli* B was prepared as described previously[1]. The purified enzyme (1750 units/0.35 mg protein per ml of 0.4 M ammonium sulfate at pH 7.6) was kept frozen at −20°. Prior to use, the enzyme was diluted more than 70 times with ice-cold 0.02 M Tris–HCl (pH 7.8) in the arsenolytic method and 500 times with 0.4 M ammonium sulfate solution (pH 7.8) in the acetyl-CoA formation method. Aliquots (50 μl) from both solutions were added to the reaction mixtures. Ammonium sulfate contaminating the mixture should be less than 0.6 mM in the arsenolytic method and 6.7 mM in the acetyl-CoA formation method.

Determination of the enzyme activity. The activity of phosphotransacetylase was measured by either the CoA-dependent arsenolytic decomposition of acetyl phosphate (acetyl-*P*) or the acetyl-CoA formation from CoA and acetyl-*P*, accordingly to reaction systems. The latter method was used for the reaction systems in which the concentration of acetyl-*P* was varied over 4 mM because of a considerable substrate inhibition by acetyl-*P* at the concentrations above this limit in the former method[1].

In the arsenolytic method, the reaction mixture contained 15.8 mμmoles (5 Lipmann units) of CoA, 2 μmoles of acetyl-*P*, 25 μmoles of potassium arsenate (pH 7.8), 10 μmoles of Tris–HCl (pH 7.8), 5 μmoles of cysteine and 50 μl of the enzyme in a final volume of 0.5 ml. In the system in which the CoA concentration was varied, 10 μmoles of cysteine were used. After incubation at 25° for 10 min, the remaining amount of acetyl-*P* was determined by hydroxamic acid method II (see ref. 1).

The acetyl-CoA formation method is a modification of the method of BERGMEYER *et al.*[5]. 120 μmoles of Tris–HCl (pH 7.8), 0.5 μmole of CoA, 3 μmoles of dithiothreitol, 100 μmoles of NH_4Cl, 0.8–60 μmoles of acetyl-*P* and redistilled water were added to a cuvette with a 1-cm light path to give a volume of 2.95 ml. After standing 20 min at 22°, the reaction was started by the addition of 50 μl of the enzyme, and the increase in absorbance at 233 mμ was read every 10 sec at 22° (see ref. 1).

Determination of concentrations of effectors. Nucleotides of adenosine (ε_M 15 400), guanosine (ε_M 11 800), inosine (ε_M 7400), cytosine (ε_M 7300) and uridine (ε_M 9900), as well as NAD^+ (ε_M 18 000) and $NADP^+$ (ε_M 18 000), were determined by absorbance at 260 mμ. NADH and NADPH were determined by absorbance at 340 mμ (ε_M 6200) at pH 7. NADH was also determined by the decrease in the absorbance after the lactate dehydrogenase (EC 1.1.1.27) reaction in the presence of pyruvate[6]. Pyruvate was determined by colorimetry with 2,4-dinitrophenylhydrazine[7] and identified by the reaction of lactate dehydrogenase, in which the decrease of NADH accompanying the reduction of pyruvate was measured[6]. ADP was determined by measuring the amount of pyruvate formed from ADP and phosphoenolpyruvate by the pyruvate kinase (EC 2.7.1.40) reaction[7].

Reagents. NAD^+, $NADP^+$, NADH, acetyl-*P*, phosphoenolpyruvate, glucose 6-phosphate, crystalline phosphotransacetylase from *Clostridium kluyveri*, lactate dehydrogenase of rabbit muscle and pyruvate kinase of rabbit muscle were purchased from Boehringer. Purity of NAD^+, $NADP^+$ and NADH were 87%, 85% and 88%, respectively. NADPH was prepared from $NADP^+$ by the reaction of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and was purified chromatographically (purity, 92%). ADP, ADP-ribose and AMP were obtained from Sigma Chemical Company. Crystalline sodium pyruvate was obtained from Daiichi Pure Chemical Company.

Recrystallized preparation of ATP was a product of this company. Pure CoA was used, which was synthesized chemically in this laboratory[8]. Other materials were obtained from various commercial sources.

RESULTS

Inhibitors of phosphotransacetylase

Phosphotransacetylase of *E. coli* B has been found to be inhibited by various compounds shown in Table I. The result indicated in the table reveals a remarkable feature of inhibition of this enzyme. NADH and NADPH exerted inhibition, whereas no inhibitory action was observed with both NAD^+ and $NADP^+$. NADH had the most potent inhibitory effect among the various compounds tested. ADP and ATP also had an inhibitory effect; the former was more potent than the latter. Concerning the

TABLE I

INHIBITORY EFFECT OF VARIOUS SUBSTANCES ON PHOSPHOTRANSACETYLASE ACTIVITY

The enzyme activity was measured from the arsenolysis reaction with 1.25 units of the enzyme and with $4 \cdot 10^{-3}$ M of acetyl-*P*, in the presence of various compounds indicated in the table; conditions, see MATERIALS AND METHODS.

Substances	*Concn. (mM)*	*Inhibition (%)*
NAD^+	0.50	0
$NADP^+$	0.46	0
NADH	0.40	74
NADPH	0.58	27
AMP	1.00	2
ADP	0.50	60
ATP	0.50	37
ADP-ribose	0.55	14

inhibitory effects of ADP and ATP, the possibility of interconversion between them was unlikely to be the case because of the lack of the adenylate kinase (EC 2.7.4.3) activity in the reaction system under study as described below. Of various nucleotides tested, ITP, UTP, CTP, GTP and ADP-ribose were found to inhibit the enzyme activity, but their inhibitory strengths were less than one-half of ATP. GDP, IDP, CDP, UDP, AMP, GMP and UMP, so far being tested, did not affect the enzyme activity. From the analogous experiments, it was found that phosphotransacetylase of *Cl. kluyveri* was unaffected by NADH and ADP.

The amount of NADH added to the reaction mixture was found to remain unchanged during the reaction; 0.195 μmole and 0.197 μmole of NADH were obtained by measuring absorbance at 340 mμ before and after the reaction, respectively. This absorbance at 340 mμ after the reaction was completely removed by the lactate dehydrogenase reaction (see MATERIALS AND METHODS). There was also no difference in ADP content before and after the reaction when measured by the pyruvate kinase reaction (see MATERIALS AND METHODS). These results indicate that NADH and ADP were not involved in the transacetylation catalyzed by phosphotransacetylase of *E. coli* B.

Relationship between concentrations of the inhibitors and their inhibitory strengths is illustrated in Fig. 1. From the figure, the concentrations giving 50% inhibition were found to be 0.1 mM, 0.35 mM, 0.8 mM and 3.5 mM for NADH, ADP, ATP and NADPH, respectively, indicating that NADH was the most potent inhibitor of this enzyme and that the inhibitory effects of ADP, ATP and NADPH were 28.5%, 12.5% and 2.9% of NADH, respectively.

As shown in Fig. 2, both NADH and ADP were noncompetitive inhibitors with CoA, and their K_i values were $6.6 \cdot 10^{-5}$ M and $3.3 \cdot 10^{-4}$ M, respectively. ATP was also found to be a noncompetitive inhibitor with CoA, and its K_i was $8 \cdot 10^{-4}$ M. These results suggest that these inhibitors exerted their actions on a site different from that for CoA. Inhibitory strengths of ADP and ATP were only 20% and 8% of that by NADH, respectively.

The reaction velocity measured from the acetyl-CoA formation proceeded in the Michaelis–Menten type in respect to the acetyl-*P* concentrations, as shown in Fig. 3, and K_m was $3.5 \cdot 10^{-3}$–$4 \cdot 10^{-3}$ M. However, in the presence of $4.8 \cdot 10^{-5}$ M NADH,

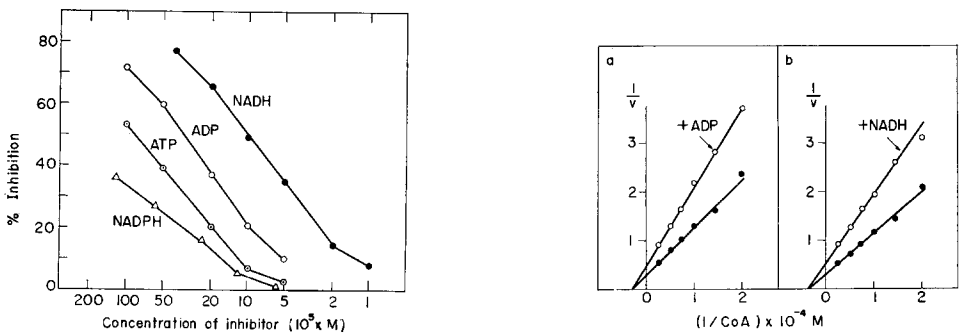


Fig. 1. Relationship between concentrations of inhibitors and their inhibitory strengths. The enzyme activity was measured from the arsenolysis reaction under the same conditions as described in the legend for Table I, except that varied concentrations of inhibitors were added to the reaction mixture.

Fig. 2. Double reciprocal plot of the phosphotransacetylase reaction with respect to the CoA concentration in the presence and the absence of inhibitor. The reaction velocity was measured from the arsenolysis reaction with $4 \cdot 10^{-3}$ M acetyl-*P*, 0.3 unit of the enzyme and with varied concentrations of CoA (see MATERIALS AND METHODS), in the presence and the absence of inhibitor. a, $2 \cdot 10^{-4}$ M ADP; b, $5 \cdot 10^{-5}$ M NADH.

a sigmoidal dependence of the reaction velocity on the acetyl-*P* concentration was found as illustrated in Fig. 3a. The maximal velocities of the reactions in the presence and the absence of NADH coincided with each other, and Hill's coefficient[9] has been calculated to be 1.60. ADP ($8.3 \cdot 10^{-5}$ M) was also found to inhibit the acetyl-CoA formation in a sigmoidal curve with Hill's coefficient of 1.42 (Fig. 3b). From the arsenolytic measurements of the reaction, similar results were obtained; K_m for acetyl-*P* was $4 \cdot 10^{-3}$ M, and Hill's coefficients were 1.50, 1.33 and 1.30 in the presence of NADH ($3.6 \cdot 10^{-5}$ M), ADP ($1.0 \cdot 10^{-4}$ M) and ATP ($1.0 \cdot 10^{-4}$ M), respectively.

No apparent inhibition of acetyl-CoA formation by ADP has been observed with 4 mM of acetyl-*P* at pH 5.8, in contrast to the above result at pH 7.8. At this acidic pH, however, K_m for acetyl-*P* was found to be $4 \cdot 10^{-4}$ M, one-tenth of the value

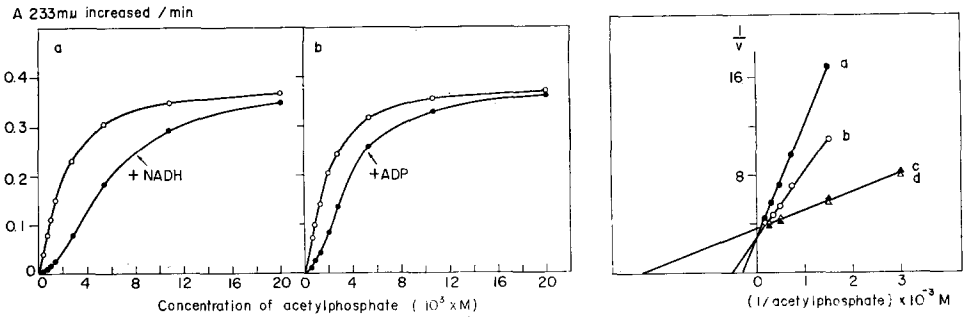


Fig. 3. Effect of inhibitor on the reaction velocity with respect to the acetyl-*P* concentrations. The enzyme (0.18 unit) was incubated at 22° in a 3-ml reaction mixture, with 0.5 μmole of CoA and with varied concentrations of acetyl-*P*, in the presence and the absence of inhibitor. The formation of acetyl-CoA was estimated from the increase in absorbance at 233 mμ (see MATERIALS AND METHODS). a, $4.8 \cdot 10^{-5}$ M NADH; b, $8.3 \cdot 10^{-5}$ M ADP.

Fig. 4. Effect of pyruvate on phosphotransacetylase activity at pH 7.8 and at pH 5.8. The enzyme reaction velocity was estimated from measuring acetyl-CoA formed, under the same conditions as described in the legend for Fig. 3, with 0.125 unit of the enzyme and varied concentrations of acetyl-*P*, in the presence and the absence of 1 mM pyruvate. The reaction mixture of pH 5.8 was prepared under the conditions described above, except that 120 μmoles of potassium acetate buffer (pH 5.8) and 0.35 unit of the enzyme were used. a, pH 7.8 without pyruvate; b, pH 7.8 with 1 mM pyruvate; c, pH 5.8 without pyruvate; d, pH 5.8 with 1 mM pyruvate.

at pH 7.8, and the inhibition by ADP was again apparent with acetyl-*P* at the concentrations of 1 mM or lower.

Stimulatory effect of pyruvate on phosphotransacetylase

Pyruvate was found to activate phosphotransacetylase of *E. coli* B. Pyruvate at 0.5 mM, 1 mM, 2 mM and 5 mM of concentrations stimulated the enzyme activity in the arsenolytic system by 12%, 20%, 30% and 34%, respectively. Although the stimulatory effect of pyruvate was not remarkable, it was interesting to observe that pyruvate repressed the inhibitory actions of NADH, ADP and ATP, as described in detail later (Figs. 5 and 6). In these experiments, α-ketoacid content in the reaction mixture determined by colorimetry with 2,4-dinitrophenylhydrazine was found to remain unchanged during the reaction and the α-ketoacid present in the incubated mixture was identified with pyruvate by the lactate dehydrogenase reaction (see MATERIALS AND METHODS). These results indicate that pyruvate was not consumed during the phosphotransacetylase reaction. Phosphotransacetylase from *Cl. kluyveri* was not affected by pyruvate at all, again supporting that pyruvate was not involved in the transacetylation.

The stimulatory mechanism of pyruvate has been further studied with the Lineweaver–Burk plots of the acetyl-CoA formation in respect to the acetyl-*P* concentration. Lines a and b in Fig. 4 represent the acetyl-CoA formation at pH 7.8, an optimum pH of phosphotransacetylase reaction, in the absence and the presence of 1 mM pyruvate, respectively. The result indicates that pyruvate activated the enzyme by lowering K_m for acetyl-*P* without a change in v_{max}. Double reciprocal plots of the arsenolysis velocity and the substrate concentration showed again that pyruvate lowered K_m for acetyl-*P* without a change in v_{max} and that it increased v_{max} without changing K_m for CoA (data are not presented). Lines c and d shown in Fig. 4 represent

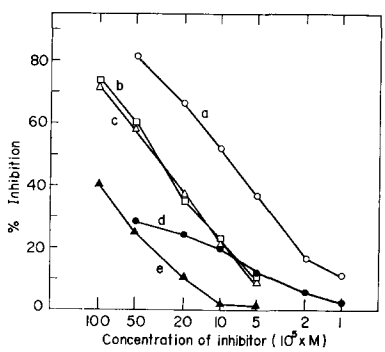
the acetyl-CoA formations at pH 5.8 in the absence and the presence of 1 mM pyruvate, respectively; K_m at this pH was $4 \cdot 10^{-4}$ M, one-tenth of the value at pH 7.8. The result indicates that the enzyme was not activated by pyruvate at this acidic pH, strongly suggesting that a stimulatory site for pyruvate was distinct from an active site of the enzyme.

There was no marked effect on the phosphotransacetylase activity of *E. coli* B from the following substances, so far being tested: α-ketoglutarate, oxaloacetate, glyoxylate, lactate, phosphoenolpyruvate, citrate, succinate, acetate, propionate, palmitate, alanine and glucose 6-phosphate.

Concomitant operation of activator and inhibitors

As described above, pyruvate activates, while NADH, ADP and ATP inhibit phosphotransacetylase of *E. coli* B. Effect of concomitant presence of these activator and inhibitors on the enzyme activity was studied, and it was found that pyruvate repressed the inhibitory action of the inhibitors and, therefore, that the stimulation by pyruvate was more effective on phosphotransacetylase inhibited by NADH, ADP or ATP. These results were indicated in Fig. 5 and Fig. 6.

Curves a and d in Fig. 5 represent the inhibitory actions of NADH in the absence and the presence of 2 mM pyruvate, respectively. This result clearly indicates that pyruvate reduced inhibitory strength of NADH. The enzyme preparation used in this



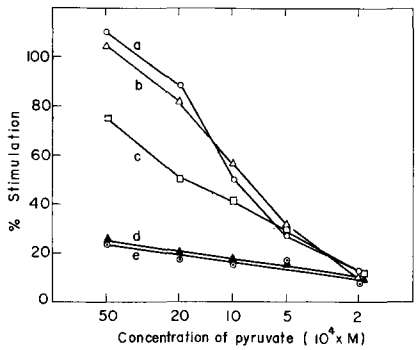


Fig. 5. Inhibitory effects of NADH and ADP in the presence of pyruvate. Conditions were the same as those described for Table I, except that the arsenolysis reaction at varied concentrations of inhibitor was carried out in the presence and the absence of pyruvate or α-ketoglutarate. a, NADH; b, ADP; c, ADP + 2 mM α-ketoglutarate; d, NADH + 2 mM pyruvate; e, ADP + 2 mM pyruvate.

Fig. 6. Stimulatory effect of pyruvate in the presence of inhibitor. Assay conditions were the same with those described for Fig. 5, except that the enzyme was incubated with varied concentrations of pyruvate in the presence and the absence of inhibitors. a, +0.5 mM ADP; b, +0.1 mM NADH; c, +1 mM ATP; d, +0.5 mM GDP; e, no added inhibitor.

experiment did not contain lactate dehydrogenase, thus no oxidation of NADH should occur in the presence of pyruvate. Curves b and e represent the effects of ADP in the absence and the presence of 2 mM pyruvate, respectively, also indicating that pyruvate repressed the inhibitory action of ADP. From the similar experiment, inhibitory action of ATP was found to be repressed by pyruvate. As a control experiment, the effect of ADP has been examined in the presence of 2 mM α-ketoglutarate,

a substance which did not affect the phosphotransacetylase activity. As represented by Curve c, the inhibitory action of ADP was unaffected by this compound.

Fig. 6 shows the stimulatory actions of pyruvate at varied concentrations in the presence and the absence of inhibitors. Curve e represents the stimulatory action of pyruvate alone, while Curves a, b and c indicate the stimulatory actions of pyruvate on the enzyme activity in the presence of ADP, NADH and ATP, respectively. These results indicate that the activation by pyruvate was more effective in the presence than in the absence of the inhibitors. As a control, stimulatory rate by pyruvate has been measured in the presence of GDP, a substance which did not affect the enzyme activity. The result, as represented by Curve d, indicates that the stimulation of pyruvate was not affected by the presence of GDP.

The stimulatory mechanism of pyruvate in the presence of NADH has been further investigated kinetically. The experiments involve comparison of the enzyme reaction at varied concentrations of acetyl-*P* in the presence of NADH with that in the concomitant presence of NADH and pyruvate. Fig. 7 shows the arsenolysis reaction in the presence and the absence of effectors. From the figure it was found

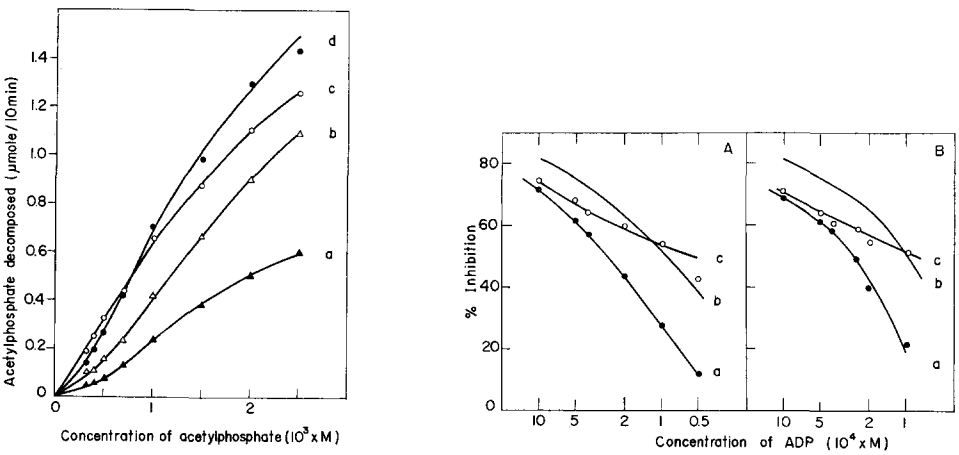


Fig. 7. Effect of pyruvate on phosphotransacetylase activity in the presence of NADH. The enzyme reaction was carried out at 25° for 10 min in a 1.0-ml arsenolysis reaction mixture with varied concentrations of acetyl-*P*, which contained 15.8 mμmoles of CoA, 5 μmoles of cysteine, 20 μmoles of Tris–HCl (pH 7.8), 50 μmoles of potassium arsenate (pH 7.8) and 0.65 unit of the enzyme in the presence and the absence of effectors. a, +0.05 mM NADH; b, +0.05 mM NADH and 0.5 mM pyruvate; c, no added effector; d, +0.05 mM NADH and 2 mM pyruvate.

Fig. 8. Inhibitory effect of ADP in the presence of NADH and of ATP. Inhibitory rate of ADP at its varied concentrations was estimated in the presence and the absence of NADH or of ATP. The enzyme reaction was carried out in 0.5 ml of arsenolysis reaction mixture containing $4 \cdot 10^{-3}$ M acetyl-*P* (see MATERIALS AND METHODS). Fig. 8A, inhibition of ADP in the presence of 0.05 mM NADH. a, measured inhibitory rate of ADP alone; b, theoretical noncompetition of ADP with NADH (0.05 mM NADH inhibited the enzyme activity by 32%); c, theoretical competition of ADP with NADH (K_i's for ADP and for NADH were $3.3 \cdot 10^{-4}$ M and $6.6 \cdot 10^{-5}$ M, respectively); ○, measured inhibitory rate of ADP in the presence of NADH. Fig. 8B, inhibition of ADP in the presence of 0.5 mM ATP. a, measured inhibitory rate of ADP alone; b, theoretical noncompetition of ADP with ATP (0.5 mM ATP inhibited the enzyme activity by 37%); c, theoretical competition of ADP with ATP (K_i for ATP was $8 \cdot 10^{-4}$ M); ○, measured inhibitory rate of ADP in the presence of ATP.

that the reaction velocity in the presence of NADH was enhanced by 80% and 150% by the addition of 0.5 mM and 2 mM of pyruvate, respectively. However, all these three reactions proceeded in sigmoidal curves with a close Hill's coefficient; the values calculated from Curves a, b and d were 1.50, 1.50 and 1.48, respectively, assuming that maximal velocities of these reactions were coincided with that of the reaction in the absence of the effector. The above result which showed that the sigmoidal nature of the reaction caused by NADH was not overcome by the stimulatory action of pyruvate, suggests that inhibition by NADH and activation by pyruvate might be based on mechanisms different from each other.

Relationship between respective inhibitory actions of NADH, ADP and ATP have been investigated by comparing inhibition rate by a mixture of two inhibitors with that by one of them. If two inhibitors compete for the same site, inhibitory rate of a mixture of the two inhibitors would be affected, depending on their affinity constants ($1/K_i$). The strength of inhibition due to a mixture of two inhibitors (concentrations, c_1 and c_2, respectively) should be equivalent to that due to either one or the other alone at the concentration of $c_1 + c_2 (K_{i1}/K_{i2})$ or $c_2 + c_1 (K_{i2}/K_{i1})$, respectively. This calculation gives a theoretical competition curve. On the other hand, if two inhibitors affect the enzyme independently, inhibitory rate due to a mixture of the two inhibitors (inhibition rates I_1 and I_2, respectively) should be equivalent to $I_1 \times I_2$. This calculation provides a theoretical noncompetition curve. Fig. 8A shows a relationship between inhibition of ADP and that of NADH. Curve a indicates the measured inhibitory strength of ADP at its varied concentrations. Curves c and b represent the theoretical competition curve and the theoretical noncompetition curve of ADP in the presence of 0.05 mM NADH, respectively. Open circles in the figure indicate the measured inhibitory strengths of the mixtures of ADP and NADH, which were almost consistent with the competition curve. From the similar experiments, ATP was also found to compete with ADP (see Fig. 8B).

Stability of phosphotransacetylase in the presence of effectors

Phosphotransacetylase of *E. coli* B was easily inactivated by dilution and by *p*-chloromercuribenzoate (PCMB)[1]. These two types of inactivation were different from each other in the following respects. Inactivation of the enzyme by dilution was not protected by mercaptoethanol but was completely protected by 0.2 M ammonium sulfate. PCMB caused more rapid inactivation of the enzyme than dilution and stabilizing effect of ammonium sulfate on the enzyme was not complete in the presence of PCMB.

Stability of the enzyme has been studied in the presence of 20 mM pyruvate and 1 mM ADP, respectively, in the two types of inactivation systems described above. Pyruvate was found to protect the enzyme against inactivation by dilution, although less effective than 0.2 M ammonium sulfate, while neither protection nor acceleration of the inactivation was found with ADP. On the other hand, both pyruvate and ADP considerably protected the enzyme against inactivation by PCMB. These results suggest that respective conformational changes in the enzyme molecule might have occurred in the presence of pyruvate and of ADP.

DISCUSSION

Phosphotransacetylase of *E. coli* B has been found to be a regulatory enzyme which was inhibited mainly by NADH and activated by pyruvate. This enzyme appears to be included in a category of allosteric enzyme[9,10], in the sense that chemical structures of the effectors and the substrates of this enzyme were allo-steric from each other and that sites for the effectors were distinct from an active site of the enzyme as well as that the effectors were not involved in the transacetylation catalyzed by this enzyme.

Inhibitory action of NADH was highly specific, since NADPH little affected and NAD^+ and $NADP^+$ did not affect the enzyme activity (Table I). NADH was a noncompetitive inhibitor in respect to CoA with a K_i value of $6.6 \cdot 10^{-5}$ M at $4 \cdot 10^{-3}$ M of acetyl-*P* (Fig. 2) and inhibited the reaction in a sigmoidal curve of Hill's coefficient of 1.60 at $4.8 \cdot 10^{-5}$ M of NADH with respect to the acetyl-*P* concentrations (Fig. 3). ADP and ATP gave also noncompetitive inhibitions with respect to CoA and caused the sigmoidicity of the reaction in respect to acetyl-*P*. However, the inhibitory strength of ADP and that of ATP were less than that of NADH, one-quater and one-tenth of NADH, respectively (Fig. 1). ITP, UTP, CTP, GTP, NADPH and ADP-ribose also exerted slight inhibition on the enzyme activity; their inhibitory strengths were less than one-twentieth of NADH. These findings might indicate that inhibition of the enzyme is controlled mainly by NADH.

Pyruvate was found to activate the enzyme. Double reciprocal plots of the reaction velocity and the acetyl-*P* concentration showed that pyruvate lowered K_m for acetyl-*P* without a change in v_{max} (Fig. 4). Moreover, pyruvate has been shown to suppress the inhibitory action of the inhibitors (Fig. 5) and therefore to enhance quite effectively the enzyme activity which had been inhibited by NADH, ADP or ATP (Fig. 6). These findings suggest that pyruvate plays an important role in the control of phosphotransacetylase of *E. coli* B.

The inhibitors and activator of this enzyme were not consumed during the phosphotransacetylase reaction, indicating that these effectors are not involved in the transacetylation catalyzed by this enzyme. This was supported by the finding that phosphotransacetylase of *Cl. kluyveri* was unaffected by these effectors.

Sites for NADH, ADP and ATP were suggested to be distinct from an active site of the enzyme, since these inhibitors were noncompetitive with CoA and since the chemical structures of these inhibitors and those of the substrates of this enzyme were different from each other. In the previous communication[4], the author suggested that an inhibitory site for ADP and an active site of the enzyme were different. This was based on the finding that the inhibitory action of ADP was diminished at acidic pH's below 6.0 (see ref. 4). As described in this report, however, K_m for acetyl-*P* at pH 5.8 was one-tenth of the value at pH 7.8, an optimum pH, and it was found that the reaction was inhibited by ADP also at this acidic pH at the concentrations of acetyl-*P* far below saturation. For the present time, therefore, no direct evidence has been obtained as to whether an inhibitory site and an active site are distinct. On the other hand, pyruvate did not affect the enzyme activity at pH 5.8 even at lower concentrations of acetyl-*P* (Fig. 4), strongly suggesting that a stimulatory site and an active site of the enzyme were distinct.

Pyruvate has been shown to have a remarkable feature in that it quite effectively

stimulated the enzyme activity which had been inhibited by NADH, ADP or ATP. In the presence of NADH, the enzyme activity was stimulated by 80% and by 150% with pyruvate at the concentrations of 0.5 mM and 2 mM, respectively, but the sigmoidicity of the reaction was not overcome by the presence of pyruvate (Fig. 7). The result might indicate that a stimulatory site and an inhibitory site are distinct from each other on the enzyme surface.

The behavior of ADP and that of ATP in affecting the enzyme are likely similar to that of NADH in the following two respects: noncompetitivity with CoA and the occurrence of sigmoidicity of the reaction with respect to acetyl-*P*. Moreover, the inhibitory actions of these inhibitors were shown to compete with each other (Fig. 8). These findings suggest that these inhibitors interact with the same one site on the enzyme surface, although it cannot be ruled out that the enzyme has multiple, specific inhibitory sites for respective inhibitors, which are separately located on the enzyme. In the latter case, however, it has to be assumed that interactions of the inhibitors with respective inhibitory sites affect the enzyme molecule in a similar manner, as well as that one of these inhibitors affect the enzyme molecule so as to reduce the interactions of the others with their specific inhibitory sites[11], *vice versa*.

In the presence of the inhibitors, co-operativity of the reaction has been found in respect to acetyl-*P*, while the reaction was the Michaelis–Menten type with respect to the CoA concentration, as described above. It seems worthy mentioning here, therefore, that as far as phosphotransacetylase of *E. coli* B is concerned, the occurrence of sigmoidal nature of the reaction can not be explained by the symmetry model of MONOD *et al.*[12].

Phosphotransacetylase of *Cl. kluyveri* was unaffected by NADH, ADP and pyruvate, those which were found as allosteric effectors of the enzyme of *E. coli* B. This difference in characters between these two enzymes is likely to be related to the difference in their molecular weight; the former had $4 \cdot 10^4$–$6 \cdot 10^4$ while the latter had $1.6 \cdot 10^5$–$2.5 \cdot 10^5$, respectively[1]. Between these two enzymes, several other differences in properties were found which involve behavior in acid ammonium sulfate fractionation and K_m's for CoA and for acetyl-*P* (ref. 1). The reason for these differences in properties might be related to different biological role of this enzyme in *E. coli* cell and in *Cl. kluyveri* cell.

From the observation involving the control by pyruvate and NADH *in vitro*, it might be reasonable to assume that phosphotransacetylase activity is regulated by the tricarboxylic acid cycle or the enzyme regulates in part the cycle in *E. coli* cell through controlling the acetyl-CoA formation.

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