Regulation of the Activity of the Pyruvate Dehydrogenase Complex of *Escherichia coli**

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ABSTRACT: The activity of the *Escherichia coli* pyruvate dehydrogenase complex is inhibited by guanosine triphosphate and by acetyl coenzyme A. The enzyme complex was not inhibited by adenosine triphosphate, cytidine triphosphate, or uridine triphosphate under the conditions used. The inhibition by guanosine triphosphate is reversed specifically by guanosine diphosphate. Acetyl coenzyme A is competitive with pyruvate, whereas guanosine triphosphate is noncompetitive with pyruvate. Guanosine triphosphate and acetyl coenzyme A appear to act at separate and independent sites on the pyruvate dehydrogenase component of the enzyme complex. Nucleoside monophosphates (adenosine 5′-phosphate, cytidine 5′-phosphate, and guanosine 5′-phosphate) and, to a lesser extent, guanosine diphosphate, adenosine diphosphate, and inorganic orthophosphate, reverse the inhibition by acetyl coenzyme A. These nucleoside mono- and diphosphates and phosphate ions appear to act at a common site on pyruvate dehydrogenase. Whether this latter site is identical with the regulatory site that interacts with acetyl coenzyme A, or is a separate regulatory site which, in turn, modifies the acetyl coenzyme A site, remains to be determined.

The *Escherichia coli* pyruvate dehydrogenase complex catalyzes a coordinated sequence of reactions which can be represented by overall reaction 1. The complex consists of three enzymes, pyruvate dehydrogenase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase (a flavoprotein), which act sequentially in that order (Koike *et al.*, 1963).

$$\text{pyruvate} + \text{CoA} + \text{DPN}^+ \longrightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{DPNH} + \text{H}^+ \quad (1)$$

Previous reports indicated that the pyruvate dehydrogenase complex from pig heart (Garland and Randle, 1964) and from *E. coli* (Hansen and Henning, 1966) is inhibited by acetyl-CoA. Schwartz *et al.* (1968) demonstrated that the inhibitory effect of acetyl-CoA is exerted, at least in part, on the pyruvate dehydrogenase component of the *E. coli* complex. The activity of the *E. coli* pyruvate dehydrogenase is also affected by nucleotides (Schwartz and Reed, 1968; Shen *et al.*, 1968; Schwartz, 1969). The present communication is a detailed report of the effects of acetyl-CoA, nucleotides, and phosphate ions on the activity of the *E. coli* pyruvate dehydrogenase complex. Interrelationships of these effectors are also described.

Experimental Section

Materials. Nucleotides and acetyl-CoA were obtained from P-L Biochemicals. *N*-Tris(hydroxymethyl)methylglycine was purchased from Calbiochem, and enzyme grade ammonium sulfate was obtained from Mann. All other chemicals were analytical grade.

The pyruvate dehydrogenase complex was isolated from *E. coli*, Crookes strain, which was grown in medium containing either glucose (Reed and Willms, 1966) or sodium pyruvate (Reed and Mukherjee, 1969) as the major carbon source. The enzyme complex was purified by precipitation with protamine, ultracentrifugation, and isoelectric precipitation as described previously (Koike *et al.*, 1960; Reed and Willms, 1966). Yeast ribonucleic acid was used to remove protamine (Reed and Mukherjee, 1969). To remove traces of RNA, the highly purified preparations of the pyruvate dehydrogenase complex were subjected either to gel filtration on Sephadex G-50 or to fractionation with solid ammonium sulfate. The enzyme complex precipitated between 0.40 and 0.48 saturation. The specific activity of these preparations was about 18 in the DPN-reduction assay (see below).

To eliminate the possibility that protein-bound protamine or RNA affected the response to modulators, the enzyme complex was also purified by a different procedure. The cell-free *E. coli* extract (Reed and Willms, 1966) was centrifuged for 2.5 hr at 144,000*g* in the No. 40 rotor of a Spinco Model L ultracentrifuge. The pellet was dissolved in 0.05 M potassium phosphate buffer, pH 7.0, and the solution was clarified by centrifugation for 20 min at 18,000*g*. This centrifugation cycle was repeated, and the clear, yellow solution was fractionated with saturated ammonium sulfate, pH 7.5, to separate the pyruvate and α-ketoglutarate dehydrogenase complexes. The former complex was collected between 0.46 and 0.52 saturation. Ammonium sulfate was removed by extensive dialysis against 0.05 M phosphate buffer, pH 7.0, containing 0.5 mM EDTA. The specific activity of this preparation was about 12 in the DPN-reduction assay.

Methods. The overall activity of the pyruvate dehydrogenase complex (reaction 1) was determined by monitoring DPNH formation at 340 mμ with a Gilford recording spectrophotometer at 25°. The standard assay mixture contained 50 μmoles of potassium phosphate buffer, pH 7.9, 0.5 μmole of thiamine pyrophosphate, 5 μmoles of magnesium chloride, 2.0 μmoles

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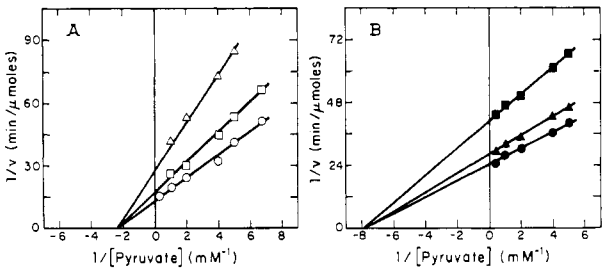

FIGURE 1: Effect of GTP on pyruvate dehydrogenase activity as a function of pyruvate concentration. (A) Activity of the pyruvate dehydrogenase complex was determined with DPN as electron acceptor as described under Methods. The concentrations of GTP were: 0 (○-○), 0.05 mM (□-□), and 0.25 mM (△-△). (B) Activity of the enzyme complex was determined with ferricyanide as electron acceptor. The assay mixtures contained 30 μmoles of potassium phosphate buffer, pH 7.5, in addition to Tricine buffer (see Methods). The concentrations of GTP were: 0 (●-●), 0.38 mM (▲-▲), and 1.0 mM (■-■).

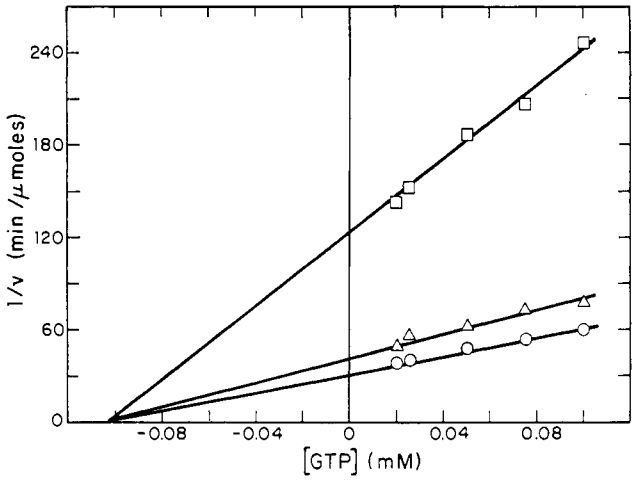

FIGURE 2: Determination of K_i (apparent) for GTP according to the method of Dixon (1953). Activity of the pyruvate dehydrogenase complex was determined in the DPN-reduction assay. The assay mixtures contained 50 μmoles of Tricine buffer, pH 7.7, instead of phosphate buffer. The concentrations of pyruvate were: 5 mM (○-○), 1 mM (△-△), and 0.1 mM (□-□).

of DPN, 0.13 μmole of CoA, 1.2 μmoles of cysteine hydrochloride, 5 μmoles of potassium pyruvate, and about 3 μg of enzyme complex in a total volume of 1.0 ml. The reaction was initiated by addition of enzyme. Modifications of the assay components and conditions are noted in the text. Activity is expressed as micromoles of DPNH formed per minute and is based on the initial rate.

The activity of the pyruvate dehydrogenase component of the enzyme complex was determined by monitoring the reduction of ferricyanide (reaction 2) at 420 mμ and 25°.

$$CH_3COCO_2H + 2\ Fe(CN)_6^{3-} + H_2O \longrightarrow CH_3CO_2H + CO_2 + 2\ Fe(CN)_6^{4-} + 2\ H^+ \quad (2)$$

The assay mixture contained 15 μmoles of Tricine[1] buffer,

[1] Abbreviation used is: Tricine, *N*-tris(hydroxymethyl)methylglycine.

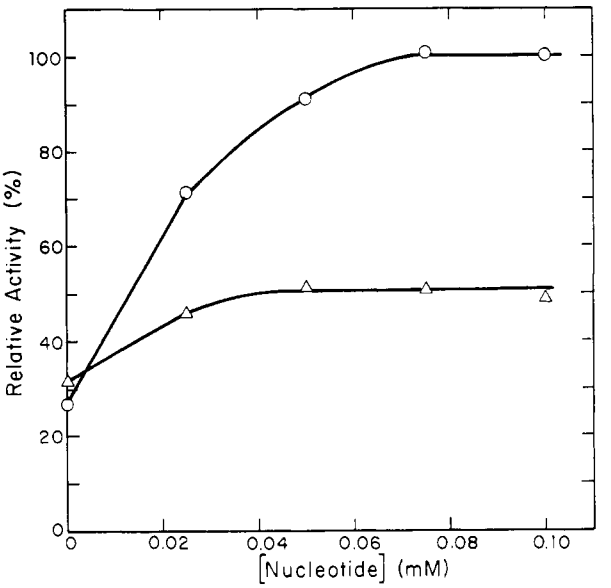

FIGURE 3: Reversal of GTP inhibition by GDP. Activity of the pyruvate dehydrogenase complex was determined in the DPN-reduction assay. The assay mixtures contained 0.5 μmole of GTP, the designated amounts of GDP (○-○) or GMP (△-△), and a modified buffer system consisting of 50 μmoles of Tricine buffer, pH 8.0, and 14 μmoles of phosphate buffer, pH 7.0. Other components and conditions were as described under Methods. The control sample (relative activity 100%) contained no nucleotides.

pH 7.7, 5 μmoles of potassium pyruvate, 10 μmoles of magnesium chloride, 0.1 μmole of thiamine pyrophosphate, 1.8 μmoles of potassium ferricyanide, and about 200 μg of the pyruvate dehydrogenase complex in a total volume of 1.0 ml. Modifications of the assay components and conditions are given in the text. Activity is expressed as micromoles of pyruvate oxidized per minute and is based on the initial rate. The activity of pyruvate dehydrogenase was also determined by measuring the rate of $[^{14}C]CO_2$ evolution with [1-^{14}C]-pyruvate as the substrate (Schwartz and Reed, 1970). Protein was determined by the biuret method (Gornall *et al.*, 1949), with crystalline bovine serum albumin as the standard.

Results

Inhibition of Pyruvate Dehydrogenase Complex by GTP. Previous studies (Schwartz *et al.*, 1968) demonstrated that the overall activity (reaction 1) of the *E. coli* pyruvate dehydrogenase complex is inhibited by acetyl-CoA and that this inhibition is competitive with respect to pyruvate. The data also indicated that the principal site of this inhibition is the pyruvate dehydrogenase component of the enzyme complex. Further investigation revealed that GTP inhibited the overall activity of the pyruvate dehydrogenase complex[2] (Figure 1A), and that this inhibition, in contrast to that observed with acetyl-CoA, is noncompetitive with respect to

[2] The preparations of pyruvate dehydrogenase complex used in these experiments were treated as described under Materials to remove RNA and protamine, or were obtained by the alternate purification procedure, which does not involve use of protamine or RNA. These latter materials tended to mask the GTP inhibition.

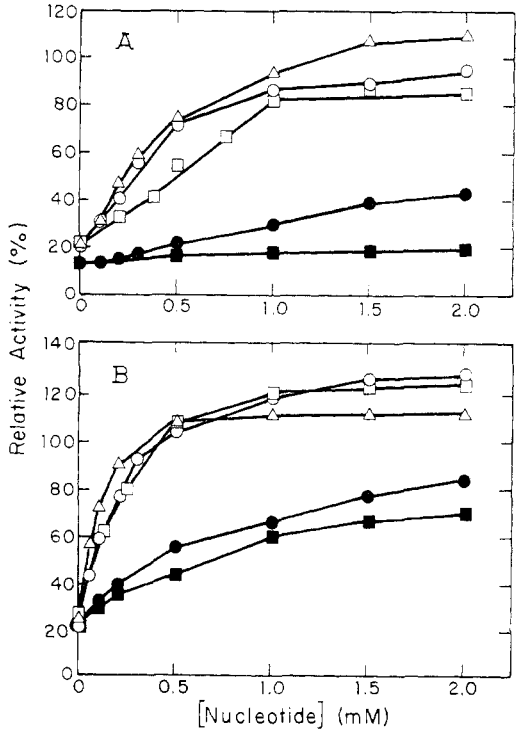

FIGURE 4: Effect of nucleoside mono- and diphosphates on inhibition of pyruvate dehydrogenase by acetyl-CoA. (A) Activity of the pyruvate dehydrogenase complex was determined with ferricyanide as electron acceptor. The assay mixtures contained 0.1 μmole of potassium pyruvate, 0.2 μmole of acetyl-CoA, and concentrations of AMP (□-□), CMP (Δ-Δ), GMP (○-○), ADP (■-■), or GDP (●-●) as indicated. (B) Activity of the enzyme complex was determined with DPN as electron acceptor. The assay mixtures contained 50 μmoles of Tricine buffer, pH 7.8, instead of phosphate buffer, 0.1 μmole of pyruvate, 0.1 μmole of acetyl-CoA, and the nucleotides designated in A. Other components and conditions in both sets of experiments were as described under Methods. The control samples contained neither acetyl-CoA nor nucleotides, and the relative activities of these samples are designated as 100%.

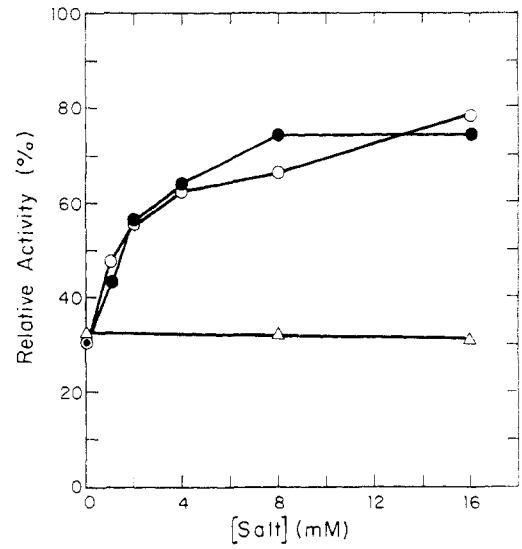

FIGURE 5: Effect of phosphate ions on inhibition of pyruvate dehydrogenase by acetyl-CoA. Activity of the pyruvate dehydrogenase complex was determined in the DPN-reduction assay. The assay mixtures contained 50 μmoles of Tricine buffer, pH 7.6, instead of phosphate buffer, 0.065 μmole of CoA, 0.05 μmole of potassium pyruvate, and the concentrations of potassium phosphate buffer, pH 7.6 (●-●), sodium phosphate buffer, pH 7.6 (○-○), or potassium chloride (Δ-Δ) as indicated. Other components and conditions were as described under Methods.

pyruvate. GTP also inhibited the activity of the enzyme complex in the ferricyanide-reduction assay (reaction 2), which is specific for the pyruvate dehydrogenase component (Koike *et al.*, 1963), and this inhibition was noncompetitive with respect to pyruvate (Figure 1B). These data indicate that GTP, like acetyl-CoA, acts on the pyruvate dehydrogenase component of the enzyme complex. However, the two effectors apparently act at different sites on the enzyme. Under the conditions used in the DPN-reduction assay, the apparent K_i for GTP was found to be 0.1 mM (Figure 2).

The inhibition by GTP was specific; ATP, CTP, and UTP were not inhibitory under the conditions used. Variation in the concentration of Mg^{2+} from 1 to 10 mM did not affect the results. The inhibition by GTP was reversed by GDP (Figure 3). GMP had only a slight effect, and ADP was ineffective.

Effect of Nucleoside Mono- and Diphosphates on Inhibition of the Pyruvate Dehydrogenase Complex by Acetyl-CoA. Preliminary experiments (Schwartz and Reed, 1968) indicated that nucleoside monophosphates and GDP, at a concentration of about 0.5 mM, stimulated the activity of pyruvate dehydrogenase about twofold in the ferricyanide-reduction assay. It was therefore of interest to determine the effect of these nucleotides on the inhibition by acetyl-CoA. Since the latter inhibition is competitive with respect to pyruvate, a relatively low concentration of pyruvate (0.05–0.1 mM) was used in these experiments. The nucleoside monophosphates AMP, CMP, and GMP reversed the inhibition by acetyl-CoA (Figure 4A). The lack of specificity suggests that nucleoside monophosphates act at a common site on the enzyme. GDP showed slight stimulation, and ADP and CDP were ineffective.

A nonspecific reversal of acetyl-CoA inhibition by nucleoside monophosphates was also observed in the DPN-reduction assay (Figure 4B). The apparent K_m for these nucleotides was about 0.1 mM. GDP and ADP showed slight stimulation, which is due, at least in part, to the presence of a small amount of the corresponding nucleoside monophosphate.[3]

Effect of Inorganic Orthophosphate on Inhibition by Acetyl-CoA. During the course of these experiments it was observed that potassium phosphate stimulated the activity of the acetyl-CoA-inhibited complex. That this effect was due to phosphate ions is indicated by the data shown in Figure 5. Sodium and potassium phosphates were equally effective, whereas equivalent concentrations of potassium chloride were ineffective. Inorganic pyrophosphate had no effect. Phosphate ions did not affect the activity of the uninhibited pyruvate dehydrogenase complex; the initial rate of DPN reduction was the same in Tricine and phosphate buffers at pH 7.85. The similarity of the effect of nucleoside monophosphates and

[3] Analysis of the GDP and ADP preparations by means of thin-layer chromatography (Randerath, 1966) showed the presence of about 5% of the corresponding nucleoside monophosphates. This amount of contaminant could account for about 50% of the stimulation observed with the nucleoside diphosphate preparations.

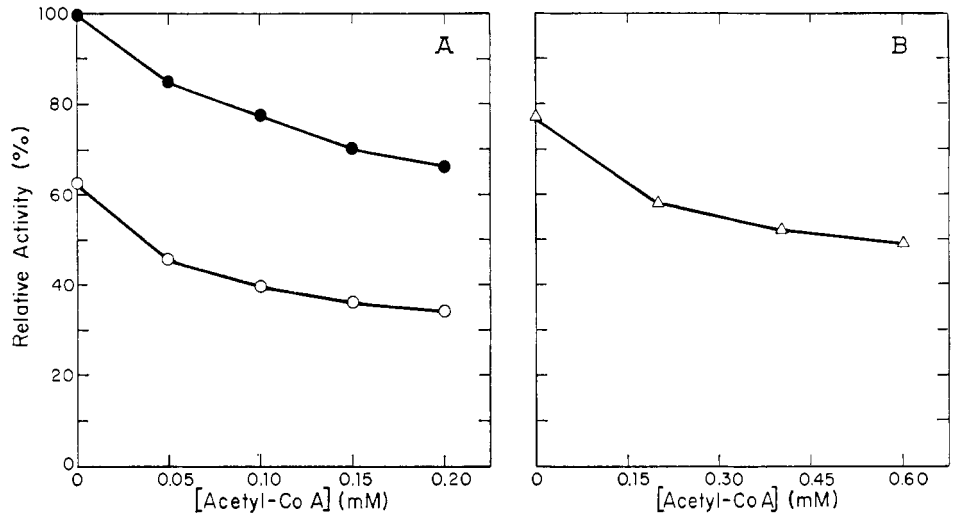

FIGURE 6: Effects of GTP and acetyl-CoA on pyruvate dehydrogenase activity. (A) Activity of the pyruvate dehydrogenase complex was determined in the DPN-reduction assay. The assay mixtures contained 80 μmoles of potassium phosphate buffer, pH 7.8, 5 μmoles of Mg^{2+}, 2 μmoles of DPN, 0.4 μmole of thiamine pyrophosphate, the indicated concentrations of acetyl-CoA, 2 μg of enzyme complex, and no GTP (●-●) or 0.5 μmole of GTP (○-○) in a total volume of 1.0 ml. Reaction was initiated by the simultaneous addition of 0.065 μmole of CoA and 0.5 μmole of pyruvate. (B) Activity of the pyruvate dehydrogenase component was determined by measuring the rate of $[^{14}C]CO_2$ evolution from [1-^{14}C]pyruvate (Schwartz and Reed, 1970). The reaction mixtures contained 10 μmoles of potassium phosphate buffer, pH 7.4, 2 μmoles of Mg^{2+}, 0.1 μmole of thiamine pyrophosphate, 0.15 mg of bovine serum albumin, 234 μg of enzyme complex, 0.19 μmole of [1-^{14}C]-pyruvate, 1 μmole of GTP, and the indicated concentrations of acetyl-CoA in a total volume of 0.42 ml. The reaction was allowed to proceed for 3 min at 23°. The control sample, which contained neither acetyl-CoA nor GTP, was assigned a relative rate of 100%.

phosphate ions suggests that these modifiers act at a common site on pyruvate dehydrogenase.

Effect of GTP on Inhibition by Acetyl-CoA. GTP did not affect the inhibition of pyruvate dehydrogenase produced by acetyl-CoA (Figure 6). In fact, the two inhibitors appeared to act independently of each other when the activity of either the enzyme complex or its pyruvate dehydrogenase component was measured. To avoid effects of small amounts of phosphate ions introduced with the reagents or produced by hydrolysis, these experiments were performed in phosphate buffer. Use of phosphate buffer, in turn, required higher concentrations of acetyl-CoA to inhibit the enzyme complex.

Response to Energy Charge. During the course of this investigation Shen *et al.* (1968) reported that the overall activity (reaction 1) of the *E. coli* pyruvate dehydrogenase complex is modulated by the energy charge of the adenylate pool and the concentration of the feedback inhibitor acetyl-CoA. According to these investigators the activity of the enzyme complex decreased with increasing energy charge, and this effect was maximal in the presence of acetyl-CoA. The experiments were conducted at a low level (0.05 mM) of pyruvate and in the absence of phosphate ions, *i.e.*, in 0.05 M Tris buffer, pH 7.6. Similar experiments have been performed in this laboratory, and typical results are presented in Figure 7A. Tricine buffer was used instead of Tris buffer, since a gradual and irreversible inactivation of pyruvate dehydrogenase occurs in Tris buffer. The effect on enzyme activity of variation of the energy charge of the adenylate system in the presence of acetyl-CoA is similar to that reported by Shen *et al.* (1968). However, this effect is not specific for the adenine nucleotides. Similar results were obtained with cytosine and guanine nucleotides. Variation of the Mg^{2+} concentration between 5 and 11 mM did not significantly affect the results. When phosphate buffer was used instead of Tricine buffer, the effect of variation of energy charge in the presence of acetyl-CoA was markedly decreased (Figure 7B). These results reflect the diminished inhibition of pyruvate dehydrogenase activity by acetyl-CoA in the presence of phosphate ions (*cf.* Figure 5). These data indicate that at low pyruvate concentration and in the absence of phosphate ions acetyl-CoA strongly inhibits pyruvate dehydrogenase activity. Nucleoside monophosphates and, to a lesser extent, nucleoside diphosphates and phosphate ions reverse this inhibition.

Discussion

The data reported in this communication indicate that GTP is an effective inhibitor (apparent K_i, about 0.1 mM) of the *E. coli* pyruvate dehydrogenase complex and that the site of this inhibition is the pyruvate dehydrogenase component of the enzyme complex. Pyruvate dehydrogenase catalyzes the initial and, apparently, rate-limiting step in pyruvate oxidation (Reed and Cox, 1966). Under the conditions of these experiments, the enzyme was not inhibited by ATP, CTP, or UTP. The inhibition by GTP was reversed specifically by GDP. Equilibrium binding studies indicate that 2 moles of GDP are bound per 183,000 daltons of apopyruvate dehydrogenase (E. R. Schwartz and L. J. Reed, 1969, unpublished data). These data suggest that the activity of pyruvate dehydrogenase may be regulated in part by the GTP:GDP ratio.

Previous studies (Schwartz *et al.*, 1968) indicated that the activity of the *E. coli* pyruvate dehydrogenase is subject to feedback inhibition by acetyl-CoA and that this inhibition is competitive with respect to pyruvate. Data obtained in the present investigation indicate that nucleoside monophosphates (AMP, CMP, and GMP), at a relatively low concentra-

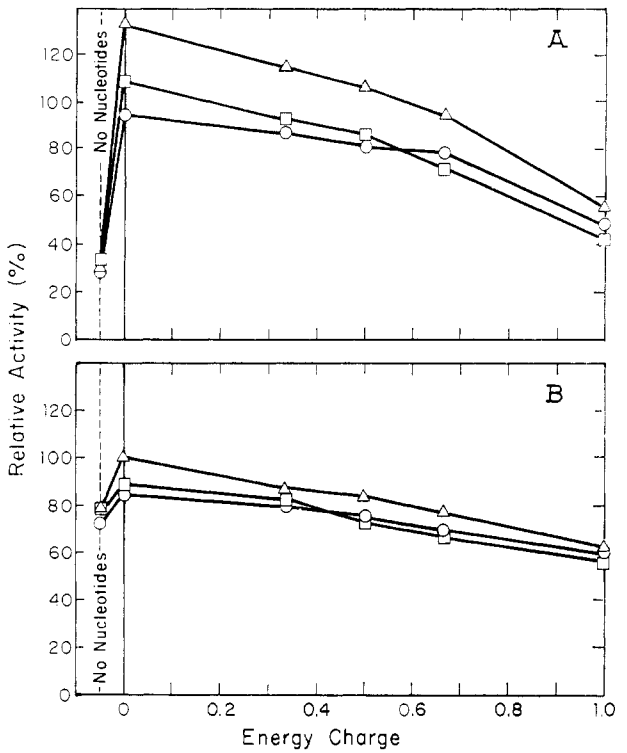

FIGURE 7: Effects of energy charge, acetyl-CoA, and phosphate ions on the activity of the pyruvate dehydrogenase complex. (A) The assay mixtures contained 75 μmoles of Tricine buffer, pH 7.9, 8 μmoles of Mg^{2+}, 1.7 μmoles of DPN, 0.5 μmole of thiamine pyrophosphate, 0.065 μmole of CoA, 0.6 μmole of cysteine hydrochloride, 0.05 μmole of potassium pyruvate, 0.1 μmole of acetyl-CoA, and a total of 5 μmoles of adenine (○-○), cytosine (△-△), or guanine (□-□) nucleotides in a final volume of 1.0 ml. Reaction was initiated by the addition of 3 μg of enzyme complex. The amounts of nucleoside mono-, di-, and triphosphates corresponding to a given energy charge were determined from the data of Atkinson (Atkinson, 1968). Rates observed in the absence of nucleotides are shown at the left side of the graph. Control samples contained neither acetyl-CoA nor nucleotides, and the relative rates obtained with these samples are designated as 100%. (B) Components and conditions were as in A, except that the buffer consisted of 50 μmoles of Tricine, pH 7.9, and 25 μmoles of potassium phosphate, pH 7.9.

tion (apparent K_m, about 0.1 mM) reverse the inhibition by acetyl-CoA. Nucleoside diphosphates (ADP and GDP) and inorganic orthophosphate also overcome the inhibition by acetyl-CoA, but substantially higher concentrations of these substances than of nucleoside monophosphates are required. It appears that the nucleoside mono- and diphosphates and inorganic phosphate act at a common site on pyruvate dehydrogenase, which is different from the site at which GTP acts.

That acetyl-CoA and GTP act at separate and, apparently, independent regulatory sites on pyruvate dehydrogenase is indicated by the following evidence. The inhibition by GTP, in contrast to that observed with acetyl-CoA, is noncompetitive with respect to pyruvate. The GTP inhibition is reversed by GDP, whereas the acetyl-CoA inhibition is reversed by nucleoside monophosphates. The extent of inhibition produced by either acetyl-CoA or GTP is not significantly affected by the presence of the other inhibitor. Whether the nucleoside monophosphates (and phosphate ions) act at the acetyl-CoA site or at a separate regulatory site which, in turn, modifies the acetyl-CoA site, remains to be determined.

The finding that the extent of inhibition by acetyl-CoA is decreased in the presence of phosphate ions explains, at least in part, the previous observation (Schwartz *et al.*, 1968) that the apparent K_i for acetyl-CoA in the oxidation of pyruvate with DPN as electron acceptor was about 40 times greater than the apparent K_i for acetyl-CoA in the oxidation of pyruvate with ferricyanide as electron acceptor. The DPN-reduction assay was carried out in 50 mM phosphate buffer, whereas the ferricyanide-reduction assay was performed in Tricine buffer.

Pyruvate occupies a central position in metabolism. Of particular importance are its oxidation to acetyl-CoA and its conversion into oxaloacetate. Oxidation of acetyl-CoA, in turn, *via* the tricarboxylic acid cycle, requires the participation of oxaloacetate and leads to the generation of ATP. The conversion of pyruvate into oxaloacetate is a major mechanism for the net synthesis of compounds of the tricarboxylic acid cycle which serve as primary biosynthetic intermediates. In *E. coli* the conversion of pyruvate into oxaloacetate is mediated by phosphoenolpyruvate synthetase and phosphoenolpyruvate carboxylase (Kornberg, 1967). Regulation of the direction of pyruvate metabolism would ensure that the needs of the cell for energy and for biosynthetic intermediates are kept in balance. There appears to be an inverse relationship in the regulation of these two pathways of pyruvate metabolism. The results reported here indicate that oxidation of pyruvate will tend to be inhibited by high levels of acetyl-CoA or when the energy charge of the nucleotide pool, as reflected by the GTP:GDP ratio, is high. Although these modulators may work in concert, they do not appear to act synergistically. On the other hand, these conditions will tend to favor conversion of pyruvate into oxaloacetate, since phosphoenolpyruvate synthetase requires ATP, and phosphoenolpyruvate carboxylase is markedly stimulated by acetyl-CoA (Kornberg, 1967).

The flavoprotein component of the *E. coli* pyruvate dehydrogenase complex is inhibited strongly by DPNH, and this inhibition is reversed by DPN (Hansen and Henning, 1966). This observation suggests that the DPNH:DPN ratio may also be an important factor in the regulation of the activity of the pyruvate dehydrogenase complex.

Studies in this laboratory with the mammalian pyruvate dehydrogenase complex have revealed (Linn *et al.*, 1969) that its activity is regulated by phosphorylation and dephosphorylation. Phosphorylation and concomitant inactivation of the pyruvate dehydrogenase component of the mammalian complex are catalyzed by an ATP-specific kinase, and dephosphorylation and concomitant reactivation are catalyzed by a phosphatase. The *E. coli* pyruvate dehydrogenase complex is not regulated by this type of control mechanism. Although the data are not presented, experiments with [γ-^{32}P]GTP, [γ-^{32}P]ATP, and preparations of the *E. coli* complex at different stages of purification gave no evidence that this enzyme complex undergoes phosphorylation.

Acknowledgments

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