Effects of Adenosine Phosphates and Nicotinamide Nucleotides on Pyruvate Carboxylase from Baker's Yeast

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1. Pyruvate carboxylase from baker's yeast is inhibited by ADP, AMP and adenosine at pH 8.0 in the presence of magnesium chloride concentrations equal to or higher than the ATP concentration. The adenine moiety is essential for the inhibitory effect. 2. In the absence of acetyl-CoA (an allosteric activator) ADP, AMP and adenosine are competitive inhibitors with respect to ATP. In the presence of acetyl-CoA, besides the effect with respect to ATP, AMP competes with acetyl-CoA, whereas ADP and adenosine are non-competitive inhibitors with respect to the activator. 3. Pyruvate carboxylase is inhibited by NADH. The inhibition is competitive with respect to acetyl-CoA and specific with respect to NADH, since NAD^+ , NADP^+ and NADPH do not affect the enzyme activity. In the absence of acetyl-CoA, NAD^+ , NADH , NADP^+ and NADPH do not inhibit pyruvate carboxylase. 4. Pyruvate carboxylase is inhibited by ADP, AMP and NADH at pH 6.5, in the presence of 12 mM-Mg^{2+} , 0.75 mM-Mn^{2+} and 0.5 mM-ATP , medium conditions similar to those existing inside the yeast cell. The ADP and NADH effects are consistent with a regulation of enzyme activity by the intracellular $[\text{ATP}]/[\text{ADP}]$ ratio and secondarily by NADH concentration. These mechanisms would supplement the already known control of yeast pyruvate carboxylase by acetyl-CoA and L-aspartate. Inhibition by AMP is less marked and its physiological role is perhaps limited.

Pyruvate carboxylase [pyruvate-carbon dioxide ligase (ADP), EC 6.4.1.1] from baker's yeast catalyses the synthesis of oxaloacetate in accordance with reaction (1):

where M^{2+} is Mg^{2+} or Mn^{2+} and M^{+} is a univalent cation (K^+ etc.). The enzyme activity can be regulated through the interaction of acetyl-CoA and L-aspartate, which act as allosteric activator and inhibitor respectively (Cazzulo & Stoppani, 1968).

The present paper deals with the effect of adenosine phosphates and nicotinamide nucleotides on the activity of yeast pyruvate carboxylase. The results obtained show that ADP can act as an enzyme inhibitor, either in conditions that are optimum for enzyme activity (pH 8; Mg^{2+} as activator) or in conditions similar to those existing inside the yeast cell (pH about 6.5; Mn^{2+} and Mg^{2+} as activators). Among other nucleotides studied, AMP and NADH were also enzyme inhibitors, but these inhibitions seem to have less physiological implication.

MATERIALS AND METHODS

Enzymes and chemicals. Pyruvate carboxylase was prepared from baker's yeast (*Saccharomyces cerevisiae*) as described by Cazzulo & Stoppani (1967). Unless stated otherwise step 6 fractions were used. *p*-Chloromercuribenzoic acid and adenosine were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Other reagents were as used by Cazzulo & Stoppani (1969).

Pyruvate carboxylase assay. In the experiments on the effect of adenosine phosphates, enzyme activity was measured with a direct spectrophotometric method (Cazzulo & Stoppani, 1969). All measurements were at 30° . The composition of the reaction mixture is stated in each case in the Results section. All measurements were made in duplicate.

In the experiments on the effect of nicotinamide nucleotides on enzyme activity, the following procedure, referred to below as the indirect spectrophotometric method, was employed. The assay mixture contained (final concentrations): tris-HCl buffer, pH 8.0, 100 mM ; KHCO_3 , 40 mM ; tris pyruvate, 8 mM ; ATP, 0.5 mM . Acetyl-CoA, MgCl_2 , pyruvate carboxylase and nicotinamide nucleotides were added as stated in the legends of the figures. The final volume was 1 ml . After incubation for 1 min . at 30° , the reaction was stopped by the addition of 0.2 ml . of $5\text{ mM-}p$ -chloromercuribenzoic acid in $50\text{ mM-glycylglycine-NaOH}$ buffer, pH 7.8 (final concn. 0.83 mM). NADH (0.44 mM) was

added immediately, and the E_{340} value was measured in a Beckman DU spectrophotometer; then 0.15 unit of malate dehydrogenase was added and E_{340} was measured again. The increase in E_{340} was taken as v . The values obtained were reproducible and in very good agreement with those determined with the direct method, provided that after *p*-chloromercuribenzoate addition subsequent operations were performed in less than 1 min.; after longer periods the values obtained were lower, probably because of the spontaneous decarboxylation of oxaloacetate.

Kinetic studies. The initial reaction velocities, v , are expressed as $\Delta E_{340}/\text{min.}$ at 30° . The values of V_{max} and K_m were obtained from double-reciprocal plots (Lineweaver & Burk, 1934). To determine the $[A]_{0.5}$ value (Atkinson, 1966) for acetyl-CoA, the value $(V - V_0)/V_0$ was employed, where V is the reaction velocity and V_0 is the reaction velocity in the absence of acetyl-CoA.

Calculation of MgADP⁻ concentrations. When necessary, an approximate calculation of MgADP⁻ concentrations was made by applying a stability constant for MgADP⁻ of 2200 M^{-1} (Burton, 1959) and the equations given by Melchior & Melchior (1958), as described for the MgATP²⁻ complex by Cazzulo & Stoppini (1969). The values obtained have only a comparative purpose and were not used for the determination of kinetic constants.

RESULTS

Effects of adenosine phosphates. Fig. 1 shows the effects of free adenine nucleotides on pyruvate carboxylase activity (magnesium chloride as activator; pH 8.0). The results can be summarized as follows. First, addition of excess of ATP (above the magnesium chloride concentration) caused a significant inhibition of enzyme activity (curves *A* and *D*). The inhibition was stronger at the lower MgATP²⁻ concentration (curve *D*). At the higher MgATP²⁻ concentration the inhibition caused by 0.25 mM-ATP was relatively small, perhaps owing to decreased dissociation of MgATP²⁻. Secondly, ADP was more effective than ATP as inhibitor of the enzyme reaction at both MgATP²⁻ concentrations (curves *B* and *E*), whereas AMP was a comparatively weaker inhibitor (curve *C*), at least in the range of concentrations employed.

Fig. 2 shows the effect of adenosine phosphates in the presence of a relative excess of magnesium chloride. In this condition ATP caused a slight activation of the enzyme reaction (curve *A*), probably owing to an increase of the MgATP²⁻ concentration. On the other hand ADP (curves *B* and *E*) and adenosine (curves *D* and *G*) produced a strong inhibition of the enzyme reaction both at 2 mM- and 0.5 mM-ATP (the latter concentration approaches the ATP concentration inside the yeast cell; Polakis & Bartley, 1966). With regard to the effect of ADP it may be assumed that the inhibition was due to the MgADP⁻ complex, since an estimate of the latter concentration, as described in the Materials and Methods section, shows that in the experimental conditions given in Fig. 2 (curve *E*)

Fig. 1. Effect of free adenine nucleotides on pyruvate carboxylase activity (assayed by the direct spectrophotometric method). The reaction mixture contained (final concentrations): tris-HCl buffer, pH 8.0, 100 mM; KHCO_3 , 40 mM; tris pyruvate, 8 mM; NADH, 0.44 mM; malate dehydrogenase (0.15 unit/ml.); acetyl-CoA, $23 \mu\text{M}$; pyruvate carboxylase (specific activity 12 units/mg.), $5.4 \mu\text{g./ml.}$ Nucleotide concentrations were as indicated on the abscissa. Curves *A* ($\circ$), *B* ($\blacktriangle$) and *C* ($\square$), enzyme activity as functions of ATP (excess), ADP and AMP concentration respectively with 2 mM-MgCl₂ and 2 mM-ATP (initial); curves *D* ($\bullet$) and *E* ($\triangle$), enzyme activity as functions of ATP (excess) and ADP concentration respectively with 0.2 mM-MgCl₂ and 0.2 mM-ATP (initial). The MgATP²⁻ initial concentrations were 1.71 mM (curves *A-C*) and 0.122 mM (curves *D* and *E*). Other experimental conditions were as described in the Materials and Methods section.

at the maximal ADP concentration employed (1 mM) the concentration of free ADP³⁻ was nearly 0.15 mM. The latter ADP³⁻ concentration caused only a slight inhibition in Fig. 1 (curve *B*). The inhibition of pyruvate carboxylase by AMP was relatively less marked (curves *C* and *F*).

The specificity of the ADP effect was investigated in the experiments described in Fig. 3. The inhibition was specific for adenosine nucleotides, since deoxy-ADP behaved exactly as ADP (curve *A*), whereas UDP, CDP, IDP, thymidine diphosphate and CMP caused no inhibition at all (curve *B*). GTP was also ineffective. Fig. 3 also shows that, in the absence of acetyl-CoA, ADP (curve *C*) and adenosine (curve *D*) again inhibited the enzyme reaction, but AMP showed no inhibitory effect (curve *E*).

Fig. 4 shows plots of $1/v$ against $1/[ATP]$ in the absence of inhibitors (curve *A*) or in the presence of 2 mM-AMP (curve *B*), 0.5 mM-ADP (curve *C*) or 1.0 mM-ADP (curve *D*). All determinations were

Fig. 2. Effects of adenine nucleotides on pyruvate carboxylase activity. Experimental conditions were as described in Fig. 1 legend, except for MgCl_2 (5 mM). Nucleotide concentrations were as indicated on the abscissa. Curves A ($\circ$), B (Δ), C ($\square$) and D (∇), enzyme activity as functions of ATP (excess), ADP, AMP and adenosine concentration respectively with 2 mM-ATP (initial); curves E ($\blacktriangle$), F ($\blacksquare$) and G ($\blacktriangledown$), enzyme activity as functions of ADP, AMP and adenosine concentration respectively with 0.5 mM-ATP (initial).

Fig. 3. Effects of adenine nucleotides on pyruvate carboxylase activity. Experimental conditions were as described in Fig. 2 legend, except for ATP (0.5 mM). Curve A, enzyme activity as a function of ADP ($\circ$) or deoxy-ADP (Δ) concentration with $23 \mu\text{M}$ -acetyl-CoA; curve B ($\square$), enzyme activity as a function of CDP (or UDP, IDP, thymidine diphosphate, GTP or CMP) concentration; curves C ($\blacktriangle$), D ($\bullet$) and E ($\blacksquare$), enzyme activity as functions of ADP, adenosine and AMP concentration respectively without acetyl-CoA.

Fig. 4. Inhibition of pyruvate carboxylase by ADP and AMP in the presence of acetyl-CoA. Experimental conditions were as described in Fig. 1 legend, except for MgCl_2 (6 mM) and pyruvate carboxylase (specific activity 14 units/mg.), ($5.4 \mu\text{g./ml.}$) ATP concentrations were as indicated on the abscissa. Curve A ($\circ$), control; curve B ($\square$), with 2 mM-AMP; curve C (Δ), with 0.5 mM-ADP; curve D ($\blacktriangle$), with 1 mM-ADP.

made in the presence of acetyl-CoA ($23 \mu\text{M}$). The straight lines obtained show that ADP changed both V_{max} and K_m values, whereas AMP changed only V_{max} , acting as a non-competitive inhibitor. Fig. 5 shows a similar experiment performed in the absence of acetyl-CoA. In contrast with the results presented in Fig. 4, both AMP (curve B) and ADP (curves C and D) behaved as strictly competitive inhibitors and almost the same occurred with adenosine (curve E). In the experimental conditions given in Figs. 4 and 5 most of ADP was present as MgADP^- . The kinetic constants obtained from the plots in Figs. 4 and 5 are presented in Table 1.

Since acetyl-CoA modified the effect of adenosine phosphates, the action of the latter on the enzyme activation by acetyl-CoA was investigated (Fig. 6). Total activities obtained are shown in curve A (control), curve B (with AMP), curve C (with ADP) and curve D (with adenosine). Curves E-G show the activities corrected for the acetyl-CoA-independent enzyme activity, as calculated with the expression $(V - V_0)/V_0$. From the latter plots it can be deduced that adenosine and the adenosine phosphates affected the enzyme activation by acetyl-CoA; the effects of ADP and adenosine were nearly the same. The double-reciprocal plots corresponding to curves E-G in Fig. 6 are presented in Fig. 7. The straight lines obtained allowed

Fig. 5. Inhibition of pyruvate carboxylase activity by ADP, AMP and adenosine in the absence of acetyl-CoA. Experimental conditions were as described in Fig. 4 legend, except for acetyl-CoA (omitted) and pyruvate carboxylase (10.8 $\mu\text{g./ml.}$). Curve A ($\circ$), control; curve B ($\square$), with 2 mM-AMP; curve C (Δ), with 0.45 mM-ADP; curve D ($\blacktriangle$), with 0.9 mM-ADP; curve E ($\bullet$), with 0.9 mM-adenosine.

Table 1. V_{max} and K_m for ATP obtained in the presence of magnesium chloride in excess and adenosine phosphates, with or without acetyl-CoA

Values were calculated from the results presented in Figs. 4 and 5.

Addition	Without acetyl-CoA		With 23 μM -acetyl-CoA	
	V_{max} ($\Delta E_{340}/\text{min.}$)	$10^3 K_m$ (M)	V_{max} ($\Delta E_{340}/\text{min.}$)	$10^3 K_m$ (M)
None	0.120	0.15	0.187	0.15
ADP (0.45 mM)	0.120	0.59	—	—
ADP (0.50 mM)	—	—	0.107	0.34
ADP (0.90 mM)	0.120	0.91	—	—
ADP (1.00 mM)	—	—	0.107	0.71
AMP (2.00 mM)	0.120	0.21	0.158	0.15
Adenosine (0.90 mM)	0.115	0.66	—	—

calculation of the $[A]_{0.5}$ values for acetyl-CoA, which increased from 9 μM without inhibitors (curve A) to 33 μM in the presence of 2 mM-AMP (curve B).

Effects of nicotinamide nucleotides. The effects of NAD^+ , NADH, NADP^+ and NADPH on pyruvate carboxylase, in the presence or in the absence of acetyl-CoA, are shown in Fig. 8. The nicotinamide nucleotide concentrations were chosen on the base of the values reported for yeast cells by Polakis & Bartley (1966). In the presence of acetyl-CoA

Fig. 6. Effects of ADP, AMP and adenosine on the activation of pyruvate carboxylase by acetyl-CoA. Experimental conditions were as described in Fig. 4 legend, except for ATP (0.5 mM), pyruvate carboxylase (specific activity 17 units/mg.), (5.8 $\mu\text{g./ml.}$) and acetyl-CoA (as indicated on the abscissa). Curve A ($\circ$), control; curve B ($\square$), with 2 mM-AMP; curve C (Δ), with 0.5 mM-ADP; curve D ($\bullet$), with 1 mM-adenosine (left-hand ordinate). Curves E ($\circ$), F ($\square$) and G (Δ and $\bullet$) represent the values corresponding to curves A, B and C (or D) respectively, corrected for the acetyl-CoA-independent activity, expressed as $(V - V_0)/V_0$ (right-hand ordinate), where V and V_0 represent enzyme activity in the presence and absence of acetyl-CoA respectively.

Fig. 7. Double-reciprocal plots obtained from the results presented in curves E-G in Fig. 6. Curve A ($\circ$), control; curve B ($\square$), with 2 mM-AMP; curve C, with 0.5 mM-ADP (Δ) or 1 mM-adenosine ($\bullet$).

NADH (curve B) produced a significant inhibition of the enzyme reaction (22% at 0.8 mM-NADH), whereas NAD^+ (curve A), NADP^+ and NADPH

Fig. 8. Effects of nicotinamide nucleotides on pyruvate carboxylase activity. Experimental conditions were as described in the Materials and Methods section (indirect spectrophotometric method of assay), except for MgCl_2 (6 mM) and pyruvate carboxylase (specific activity 17 units/mg.) (5.8 $\mu\text{g./ml.}$). Curves A (Δ), B ($\circ$) and E ($\square$), enzyme activity plotted as functions of NAD^+ , NADH and NADP^+ (or NADPH) concentration respectively in the presence of 23 μM -acetyl-CoA. Curves D ($\blacktriangle$), C ($\bullet$) and F ($\blacksquare$), enzyme activity plotted as functions of NAD^+ , NADH and NADP^+ (or NADPH) concentration respectively in the absence of acetyl-CoA.

(curve E) scarcely affected pyruvate carboxylase activity. In the absence of acetyl-CoA the nucleotides showed little or no inhibition (curves C, D and F).

Since the inhibition of pyruvate carboxylase by NADH was much more significant in the presence of acetyl-CoA, the effect of NADH on activation of the enzyme by acetyl-CoA was investigated. Fig. 9 shows that the effect of acetyl-CoA diminished in proportion to the concentration of the nicotinamide nucleotide (curves A'–C'). The corresponding double-reciprocal plots (Fig. 10, curves A–C) show that the inhibition by NADH was competitive with respect to acetyl-CoA. The $[\text{A}]_{0.5}$ value for acetyl-CoA changed from 6.2 μM in the absence of NADH (curve A) to 10 μM and 16 μM in the presence of 0.4 mM- NADH (curve B) and 0.8 mM- NADH (curve C) respectively.

Effects of NADH , ADP and AMP in the presence of magnesium chloride and manganese chloride at pH 6.5. Table 2 shows the effects of NADH , ADP and AMP on enzyme activity, when measured under conditions closer to the physiological ones, i.e. in the presence of magnesium chloride and manganese chloride at pH 6.5. The cation concentrations were chosen on the base of data summarized by Eddy

Fig. 9. Inhibition of pyruvate carboxylase by NADH . Experimental conditions were as described in Fig. 8 legend, except for MgCl_2 (2 mM) and pyruvate carboxylase (specific activity 18 units/mg.) (5.3 $\mu\text{g./ml.}$). Acetyl-CoA concentrations were as indicated on the abscissa. Curves A ($\circ$), B (Δ) and C ($\square$), enzyme activity (left-hand ordinate) as a function of acetyl-CoA concentration without NADH (control), with 0.4 mM- NADH and with 0.8 mM- NADH respectively. Curves A' ($\bullet$), B' ($\blacktriangle$) and C' ($\blacksquare$) represent the values corresponding to curves A, B and C respectively corrected for the acetyl-CoA-independent activity, expressed as $(V - V_0)/V_0$ (right-hand ordinate), where V and V_0 represent enzyme activity in the presence and absence of acetyl-CoA respectively.

(1958), and the pH value from the data of Polakis & Bartley (1965). The effects of the nucleotides were very similar to those described in the preceding paragraphs; thus the inhibition by 0.44 mM- NADH (compared with the enzyme activity with 0.14 mM- NADH) was higher in the presence of acetyl-CoA. In this experiment the direct spectrophotometric method was used, which requires addition of NADH for the coupled malate dehydrogenase reaction. At the concentration (0.14 mM) employed in the control samples NADH did not inhibit pyruvate carboxylase. The effect of ADP and AMP was tested in the presence of 0.44 mM- NADH , which is within the range of concentrations found in yeast cells (Polakis & Bartley, 1966). Both adenosine phosphates inhibited the reaction either in the presence or in the absence of acetyl-CoA, and again ADP was the more effective inhibitor.

Fig. 11 shows the variation of enzyme activity as a function of the $[\text{ATP}]/[\text{ADP}]$ ratio at a constant ATP concentration (0.5 mM). Enzyme activities are presented as percentages of the activity in the absence of ADP. For comparative purposes, values obtained at both pH 6.5 and 8.0 are presented (values at pH 8.0 were calculated on the basis of

Fig. 10. Double-reciprocal plots obtained from the results presented in Fig. 9. Curves *A* (○), *B* (Δ) and *C* (□) correspond to curves *A'*, *B'* and *C'* respectively in Fig. 9.

Table 2. *Inhibition of pyruvate carboxylase by NADH, ADP and AMP in the presence of magnesium chloride and manganese chloride at pH 6.5*

Experimental conditions were as given in Fig. 11 (curves *A* and *B*), except that the concentrations of NADH, ADP and AMP were as indicated. The percentage values for ADP and AMP refer to the enzyme activity in the presence of 0.44 mM-NADH taken as 100%.

Addition	Without acetyl-CoA		With 23 μM-acetyl-CoA	
	<i>v</i> (Δ <i>E</i> ₃₄₀ / min.)	<i>v</i> (% of control)	<i>v</i> (Δ <i>E</i> ₃₄₀ / min.)	<i>v</i> (% of control)
NADH (0.14 mM)	0.040	100	0.160	100
NADH (0.44 mM)	0.036	90 (100)	0.132	82 (100)
NADH (0.44 mM) + ADP (1 mM)	0.015	41	0.078	59
NADH (0.44 mM) + AMP (2 mM)	0.026	68	0.100	75

the results presented in Figs. 2 and 3, as stated in Fig. 11 legend). In the presence of acetyl-CoA (curves *A* and *D*) higher relative activities were obtained at pH 6.5, showing that the inhibitory effect of ADP was smaller at pH 6.5 than at pH 8.0. In the absence of acetyl-CoA (curves *B* and *C*) the inhibition by ADP was almost equal at both pH values.

DISCUSSION

It was shown in the preceding paper (Cazzulo & Stoppani, 1969) that free ATP⁴⁻ inhibits the reaction catalysed by yeast pyruvate carboxylase.

Fig. 11. Pyruvate carboxylase activity as a function of the [ATP]/[ADP] ratio. The reaction mixture (curves *A* and *B*) contained (final concentrations): potassium borate-succinate buffer, pH 6.5, 66 mM; MgCl₂, 12 mM; MnCl₂, 0.75 mM; ATP, 0.5 mM; KHCO₃, 20 mM; KCl, 20 mM; pyruvic acid, 8 mM; NADH, 0.44 mM; malate dehydrogenase, 0.15 unit/ml.; pyruvate carboxylase (step 5 fraction, specific activity 4 units/mg.), 57 μg./ml.; ADP, as required for the values of the [ATP]/[ADP] ratio indicated on the abscissa. Curve *A* (▲), with 23 μM-acetyl-CoA; curve *B* (●), without acetyl-CoA. Curve *C* (○), values calculated from Fig. 3 (curve *C*) (results at pH 8.0 in the presence of MgCl₂, without acetyl-CoA); curve *D* (Δ), values calculated from Fig. 2 (curve *E*) (experimental conditions as for curve *C*, but in the presence of 23 μM-acetyl-CoA). In the ordinate, 'relative activity' is the percentage value of the enzyme activity in the presence of ADP, with respect to non-inhibited control. Control values were 0.132 (curve *A*), 0.036 (curve *B*), 0.079 (curve *C*) and 0.105 (curve *D*).

This observation has been now confirmed and extended to free ADP and AMP (Fig. 1). In the presence of excess of magnesium chloride ADP again showed a strong inhibitory effect, whereas AMP was a poor inhibitor and ATP was ineffective (Fig. 2) even at relatively high concentrations. The effect of ADP in the presence of excess of magnesium chloride can be attributed to the complex ion MgADP⁻, since under the experimental conditions used most of ADP was in the form of this complex ion. The inhibitory effect of ADP was specific, like the nucleoside triphosphate requirement for the pyruvate carboxylase reaction (Cazzulo & Stoppani, 1967). Only deoxy-ADP was able to replace ADP, the other nucleotides tested being ineffective (Fig. 3). Similar observations have been reported by Keech & Utter (1963) for the chicken liver enzyme. The inhibition by ADP proved to be complex, since ADP affects both the enzyme's apparent affinity for the MgATP²⁻

substrate and the activation by acetyl-CoA. In fact, in the presence of acetyl-CoA ADP changed both $V_{\max}$ and K_m for ATP, whereas in the absence of activator the inhibition was strictly competitive (Figs. 4 and 5 and Table 1). With respect to the activation by acetyl-CoA, ADP showed a non-competitive inhibitory effect (Fig. 7).

The inhibition of pyruvate carboxylase by AMP was non-competitive with respect to ATP in the presence of acetyl-CoA (Fig. 4) and strictly competitive in the absence of the activator (Fig. 5). These different effects of AMP may be explained by the fact that AMP acted as a competitive inhibitor with respect to acetyl-CoA (Fig. 7).

The competitive inhibition by ADP and AMP with respect to ATP, in the absence of acetyl-CoA, involves the adenosine moiety of the nucleotides, since adenosine itself proved to be a powerful inhibitor, even stronger than AMP (Fig. 5). The enzyme presumably binds $MgADP^-$ at both the adenine and magnesium phosphate ends; this is in accord with inhibition of the enzyme reaction by either free nucleotides (Fig. 1) or free bivalent cations (Cazzulo & Stoppani, 1969).

Among the several nicotinamide nucleotides tested, NADH was the most effective inhibitor of yeast pyruvate carboxylase, and this inhibition was competitive with respect to acetyl-CoA (Fig. 10). On the other hand, NAD^+ showed only a very slight inhibitory effect, even at a high concentration (2mM). $NADP^+$ and NADPH, at the low concentrations tested, did not inhibit the enzyme reaction (Fig. 8).

It seems possible that nucleotides, like pH (Cazzulo & Stoppani, 1969), L-aspartate concentration (Palacián, de Torrontegui & Losada, 1966; Cazzulo & Stoppani, 1968) and acetyl-CoA (Ruiz-Amil, de Torrontegui, Palacián, Catalina & Losada, 1965; Cazzulo & Stoppani, 1968), may regulate pyruvate carboxylase activity within the yeast cell. With respect to the inhibitory effect of ADP, it must be borne in mind that inside the cell there is a significant concentration of ATP. Since ADP is competitive with respect to ATP, the $[ATP]/[ADP]$ ratio is more significant than the simple ADP concentration. Polakis & Bartley (1966) have shown that the $[ATP]/[ADP]$ ratio varied during yeast growth approximately from 2 (exponential phase) to 0.5 (early stationary phase). In medium conditions approaching those reported for the yeast cell (Polakis & Bartley, 1966), the variation quoted caused 50% inhibition of enzyme activity in the absence of acetyl-CoA and 30% inhibition with $23\mu M$ -acetyl-CoA (Fig. 11, curves *B* and *A* respectively). The 'energy charge' parameter (Atkinson & Walton, 1967), as applied by Stan & Schormüller (1968) to pyruvate carboxylase from *Penicillium camemberti*, seems to have less validity for the yeast

enzyme, since in the yeast cell both total adenylate concentration and the $[ATP]/[AMP]/[ADP]^2$ ratio (adenylate kinase reaction) varied markedly during growth on glucose as sole carbon source (Polakis & Bartley, 1966).

During the exponential phase of growth the conditions prevailing in the yeast cells would favour a relatively high activity of pyruvate carboxylase, since (a) the $[ATP]/[ADP]$ ratio is high and the concentrations of AMP and NADH (enzyme inhibitors) are relatively low (Polakis & Bartley, 1966), (b) the pH is about 6.5 (Polakis & Bartley, 1965) and (c) the concentration of the inhibitor L-aspartate may be relatively low because of the rapid utilization of the amino acid for biosynthetic purposes. The opposite conditions would prevail in resting yeast, where the $[ATP]/[ADP]$ ratio is low (Polakis & Bartley, 1966), the intracellular pH is lower (Polakis & Bartley, 1965) and the L-aspartate concentration may be high (Stoppani, Conches, de Favelukes & Sacerdote, 1958), all circumstances leading to a decrease in enzyme activity. The regulation of pyruvate carboxylase activity by metabolites and pH, as summarized above, is in all probability accompanied by variations in the enzyme content of the yeast cell, which increases during the exponential phase of growth (Cazzulo, Claisse & Stoppani, 1968).

During growth of baker's yeast on glucose, a main function of pyruvate carboxylase is presumably to supply the carbon skeleton for the biosynthesis of L-aspartate. This role is supported by the feedback enzyme inhibition by L-aspartate (Palacián *et al.* 1966) and by the product inhibition by ADP. Optimum enzyme activity takes place at relatively high values of the $[ATP]/[ADP]$ ratio, as can be expected for biosynthetic processes (Atkinson & Walton, 1967). Partial regulation of pyruvate carboxylase by the $[ATP]/[ADP]$ ratio has been suggested by Utter, Keech & Scrutton (1964) for the chicken liver enzyme.

The inhibition by NADH may imply a regulation of the anaplerotic function (Kornberg, 1966) of pyruvate carboxylase by the respiratory mechanisms of the cell. This assumption fits in well with the effect of NADH on the enzyme activation by acetyl-CoA, which is directly related to the anaplerotic function. Thus a high concentration of NADH would inhibit pyruvate carboxylase and thereby the synthesis of oxaloacetate. The latter effect would depress oxaloacetate concentration inside the yeast cell, and accordingly would retard the operation of the tricarboxylic acid cycle. The regulatory role of NADH has been proposed by Weitzman (1966) for citrate synthase from *Escherichia coli*. However, the inhibition of yeast pyruvate carboxylase by NADH is much less marked than that of *E. coli* citrate synthase.

The variation of AMP concentration in yeast cells growing on glucose as substrate (about 0.9–2mM; Polakis & Bartley, 1966) would cause a very limited change of pyruvate carboxylase activity (Figs. 2 and 3 and Table 2), and AMP inhibition may be of little physiological importance.

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