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Effects of Magnesium, Manganese and Adenosine Triphosphate Ions on Pyruvate Carboxylase from Baker's Yeast

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1. Pyruvate carboxylase from baker's yeast acts with either $MgATP^{2-}$ or $MnATP^{2-}$ as substrate. The optimum pH for the enzyme reaction is 8·0 with $MgATP^{2-}$ and 7·0 with $MnATP^{2-}$. 2. When the reaction velocity is plotted against $MgATP^{2-}$ (or $MnATP^{2-}$) concentration slightly sigmoid curves are obtained, either in the presence or in the absence of acetyl-CoA (an allosteric activator). In the presence of excess of free Mg^{2+} (or Mn^{2+}) the curves turn into hyperbolae, whereas in the presence of excess of free ATP^{4-} the apparent sigmoidicity of the curves increases. 3. The sigmoidicity of the plots of v against $MgATP^{2-}$ (or $MnATP^{2-}$) concentration can be explained by the inhibitory effect of free ATP^{4-}, the concentration of which, in the experimental conditions employed, is significant and varies according to the total concentration of the ATP–magnesium chloride (or ATP–manganese chloride) mixture. Free ATP^{4-} behaves as a negative modifier of yeast pyruvate carboxylase. 4. The effect of high concentrations of Mg^{2+} (or Mn^{2+}) on the kinetics of yeast pyruvate carboxylase can be explained as a de-inhibition with respect to ATP^{4-}, instead of a direct enzyme activation. 5. At pH 6·5 manganese chloride is more effective than magnesium chloride as enzyme activator even in the presence of a great excess (16-fold) of the latter. This is consistent with a significant contribution of the $MnATP^{2-}$ complex to the activity of yeast pyruvate carboxylase, in medium conditions resembling those existing inside the yeast cell (pH 6·25–6·75; 12mM-magnesium chloride and 0·75mM-manganese chloride). 6. The physiological significance of the enzyme inhibition by free ATP^{4-} is doubtful since the Mg^{2+} and Mn^{2+} concentrations reported to exist inside the yeast cell are sufficient to decrease ATP^{4-} concentrations to ineffective values.

Pyruvate carboxylase [pyruvate–carbon dioxide ligase (ADP), EC 6.4.1.1] from baker's yeast catalyses the synthesis of oxaloacetate (Losada, Cánovas & Ruiz-Amil, 1964; Gailiusis, Rinne & Benedict, 1964; Cazzulo & Stoppani, 1965) in accordance with reaction (1):

$$\text{Pyruvate} + HCO_3^- + ATP \underset{\text{acetyl-CoA}}{\overset{M^{2+}, M'^{+}}{\rightleftharpoons}} \text{oxaloacetate} + ADP + P_i \quad (1)$$

where M^{2+} is Mg^{2+} or Mn^{2+} (Losada *et al.* 1964; Cazzulo & Stoppani, 1967) and M'^{+} is a univalent cation, K^+ being the most effective (Cazzulo & Stoppani, 1967).

It has been suggested (Cazzulo & Stoppani, 1967) that the complex ion $MgATP^{2-}$ is the true substrate of yeast pyruvate carboxylase, whereas free ATP^{4-} and Mg^{2+} are inhibitors. For sheep kidney pyruvate carboxylase Keech & Barritt (1967) similarly postulated that $MgATP^{2-}$ is the actual substrate for the enzyme, with Mg^{2+} as an allosteric activator.

In the present paper a more detailed kinetic study of pyruvate carboxylase from baker's yeast is reported. This investigation was made to establish: (*a*) the behaviour of the yeast enzyme with respect to Mg^{2+} and ATP^{4-} concentrations; (*b*) the effect of the complex ion $MnATP^{2-}$ as a substrate for the reaction; (*c*) the relative enzyme activities with Mg^{2+} or Mn^{2+} under different experimental conditions, particularly with regard to the effect of pH.

MATERIALS AND METHODS

Enzymes. Pyruvate carboxylase was purified from baker's yeast (*Saccharomyces cerevisiae*) as described by Cazzulo & Stoppani (1967). Unless stated otherwise the experiments were made with step 6 fractions, specific activities of which are stated in each case. Malate dehydrogenase (specific

activity 1000 units/mg. of protein) was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Enzyme units are expressed as international units (μmoles of substrate or product/min.), and specific activities are expressed as units/mg. of protein.

Chemicals. Tris base (Trizma base), acetyl-CoA and all the nucleotides employed were purchased from Sigma Chemical Co. Pyruvic acid was prepared from sodium pyruvate (E. Merck A.-G., Darmstadt, Germany) by percolating the salt through a column of Dowex 50W (X2; H^+ form) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.).

Analytical methods. The concentrations of all the nucleotide solutions were measured spectrophotometrically. The $MgCl_2$ and $MnCl_2$ solutions were titrated complexiometrically with EDTA and Eriochrome Black T as indicator (Wilson & Wilson, 1960). Protein determinations were made spectrophotometrically (Christian & Warburg, 1939). The pH values of the reaction mixtures were determined, 3 min. after the addition of ATP, with a Metrohm Titriskop type E 166 potentiometer and a glass electrode.

Pyruvate carboxylase assay. The enzyme activity was determined spectrophotometrically, essentially by the method described by Cazzulo & Stoppani (1967). The standard assay mixture contained tris–HCl buffer, $KHCO_3$, pyruvate, NADH, malate dehydrogenase, $MgCl_2$, $MnCl_2$, ATP, acetyl-CoA and pyruvate carboxylase as stated in each case. The final volume of reaction mixtures was 1 ml. The reaction was started by adding the ATP. The enzyme activities were determined in a Beckman DU spectrophotometer at 30°, with 1 cm.-light-path silica cuvettes (Figs. 2–4) or with 0·5 cm.-light-path cuvettes (Figs. 1 and 5–10). Enzyme activities are expressed as ΔE_{340}/min. at 30°. Measurements were made in duplicate.

Kinetic studies. $V_{max.}$ and K_m values were calculated from the double-reciprocal plots (Lineweaver & Burk, 1934). When these plots were not straight lines, the $V_{max.}$ values were determined by extrapolation. The $[S]_{0.5}$ values (Atkinson, 1966) were obtained from the Hill plots, taking the value of substrate concentration required to make $\log[v/(V_{max.}-v)]=0$. The apparent n values were calculated by the least-squares method, applying the experimental results to the Hill equation (eqn. 2) in accordance with Monod, Changeux & Jacob (1963) and Atkinson (1966):

$$\log\left(\frac{v}{V_{max.}-v}\right) = n \log [S] - \log K \qquad (2)$$

Calculation of concentrations of complex ions. As pointed out by Cleland (1967), the concentrations of the different ionic species in equimolar solutions of ATP and $MgCl_2$ (or $MnCl_2$) vary with dilution. The concentrations of the complex ions $MgATP^{2-}$ and $MnATP^{2-}$ at each experimental point were calculated by employing a stability constant for $MgATP^{2-}$ of 20000 M^{-1} at pH 8·0 in tris–HCl buffer (O'Sullivan & Perrin, 1964), and a stability constant for $MnATP^{2-}$ of 279000 M^{-1} at pH values between 3 and 7·3 in tetramethylammonium bromide solutions (Perrin & Sharma, 1966). The quoted value of the stability constant for $MgATP^{2-}$ was chosen because the determinations were made in the presence of tris–HCl buffer instead of *N*-ethylmorpholine, which was used by Keech & Barritt (1967) in a similar study with sheep kidney pyruvate carboxylase. With *N*-ethylmorpholine buffer the stability constant of the $MgATP^{2-}$ complex is 73000 M^{-1} (O'Sullivan & Perrin, 1964). The calculations were made by employing the equations given by Melchior & Melchior (1958). The ionic species $HATP^{3-}$ and $KATP^{3-}$ were neglected, on the assumption that their concentrations were small in the experimental conditions used.

RESULTS

Effect of pH. Fig. 1 shows the effect of pH on pyruvate carboxylase activity in the presence of $MgATP^{2-}$ or $MnATP^{2-}$. The complex ions were employed at optimum concentrations as determined in preliminary experiments. With $MgATP^{2-}$ and acetyl-CoA (23 μM) maximal activity was at about pH 8·0 (curve *A*), in agreement with previous observations (Cazzulo & Stoppani, 1967). Omission of acetyl-CoA did not affect the maximum position (curve *B*) and the degree of activation was constant from pH 7 to pH 8·75. With the complex ion $MnATP^{2-}$ (acetyl-CoA added) the curve was completely different (Fig. 1, curve *C*): (*a*) the highest activity was obtained at about pH 7·0;

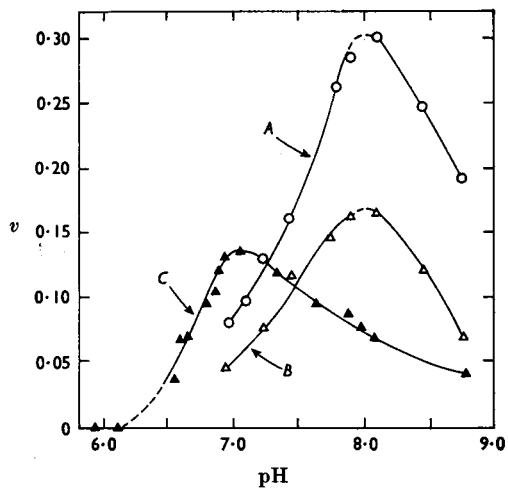

Fig. 1. Pyruvate carboxylase activity as a function of pH with $MgATP^{2-}$ and $MnATP^{2-}$ as substrates. The reaction mixture with $MgATP^{2-}$ contained (final concentrations): tris–HCl buffer (pH as indicated on the abscissa), 100 mM; $KHCO_3$, 40 mM; tris pyruvate, 8 mM; NADH, 0·44 mM; malate dehydrogenase, 0·15 unit/ml.; $MgCl_2$, 2 mM; ATP, 2 mM; pyruvate carboxylase (specific activity 12 units/mg.), 10 μg./ml. Curve *A* (○), with 23 μM-acetyl-CoA; curve *B* (△), without acetyl-CoA. The reaction mixture with $MnATP^{2-}$ [curve *C* (▲)] contained (final concentrations): sodium borate–succinate buffer, 66 mM (pH values below 7·0), or tris–HCl buffer, 100 mM (pH values above 7·0); $KHCO_3$, 20 mM; KCl, 20 mM; pyruvic acid, 8 mM; NADH, 0·44 mM; malate dehydrogenase, 0·15 unit/ml.); $MnCl_2$, 0·08 mM; ATP, 0·08 mM; acetyl-CoA, 23 μM; pyruvate carboxylase (specific activity 12 units/mg.), 10 μg./ml. The activities measured in the presence of sodium borate–succinate buffer were corrected as described in the text.

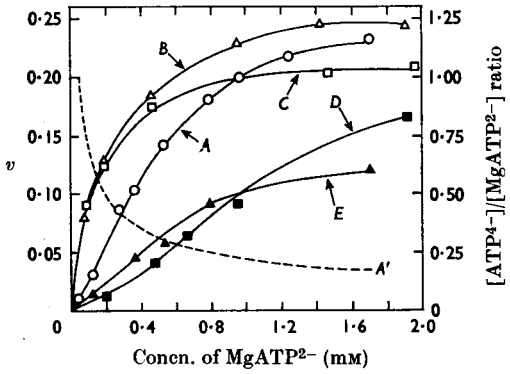

Fig. 2. Pyruvate carboxylase activity as a function of $MgATP^{2-}$ concentration. Experimental conditions were as described in Fig. 1 legend, except for pyruvate carboxylase (specific activity, 15 units/mg.), 1·8 μg./ml., and $MgATP^{2-}$ (concentrations indicated on the abscissa). Curve *A* (○), without further additions; curve *B* (△), with 1 mM-Mg^{2+} (excess); curve *C* (□), with 3 mM-Mg^{2+} (excess); curve *D* (■), with 1 mM-ATP^{4-} (excess); curve *E* (▲), as for curve *A*, but without acetyl-CoA. The broken line (curve *A'*) shows the variation of the $[ATP^{4-}]/[MgATP^{2-}]$ ratio (right-hand ordinate) corresponding to the conditions for curve *A*.

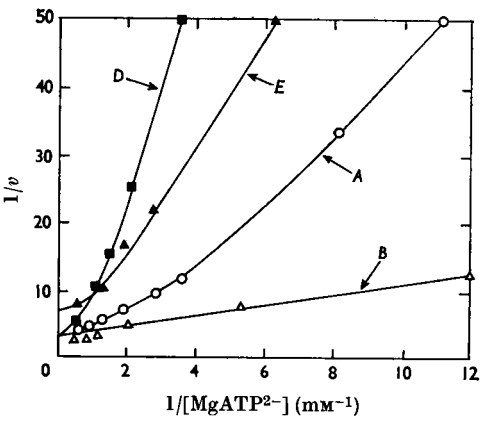

Fig. 3. Double-reciprocal plots obtained from the results presented in Fig. 2. Curves *A* (○), *B* (△), *D* (■) and *E* (▲) correspond to curves *A*, *B*, *D* and *E* respectively in Fig. 2; curve *C* is omitted.

(*b*) maximal activity was about 40% of that obtained with $MgATP^{2-}$ (curve *A*); (*c*) the peak obtained was much less symmetrical than the one obtained with $MgATP^{2-}$; (*d*) the ATP concentration used was 25-fold less than the one used with magnesium chloride. Values for enzyme activities measured in the presence of sodium borate–succinate buffer, pH 7·0 (Fig. 1, curve *C*), were corrected for the inhibitory effect of Na^+ (nearly 35%) as determined in comparison with controls in tris–hydrochloric acid buffer at pH above 7·0. On the basis of the results presented in Fig. 1, subsequent studies with the complex ions $MgATP^{2-}$ and $MnATP^{2-}$ were made at the respective pH optima, 8·0 and 7·0.

Effects of Mg^{2+} *and* ATP^{4-} *at several* $MgATP^{2-}$ *concentrations.* Fig. 2 shows the plots of initial reaction rates, *v*, against $MgATP^{2-}$ concentration. When the concentration of $MgATP^{2-}$ increased, the [free ion]/[complex ion] ratio decreased; the variation was particularly significant at the lower $MgATP^{2-}$ concentrations. The results presented can be summarized as follows. First, the control curves *A* and *E* were slightly sigmoid either in the presence of acetyl-CoA (curve *A*) or in its absence (curve *E*). Secondly, in the presence of an excess of Mg^{2+} (above the ATP concentration) the curves were hyperbolic (curves *B* and *C*). At the lower $MgATP^{2-}$ concentrations (0·1–0·5 mM) there was marked activation by Mg^{2+}, but at the higher ones (1–2 mM-$MgATP^{2-}$) the activation effect became less significant (1 mM-Mg^{2+}; curve *B*) or turned into an inhibition (3 mM-Mg^{2+}; curve *C*). Thirdly, 1 mM-ATP^{4-} in excess (above the magnesium chloride concentration) caused a strong inhibition and the sigmoidicity of the curve appeared to increase (curve *D*).

Fig. 3 shows the double-reciprocal plots corresponding to the curves presented in Fig. 2. With equimolar concentrations of magnesium chloride and ATP the curves were concave upwards, either in the presence (curve *A*) or in the absence of acetyl-CoA (curve *E*). The $V_{max.}$ without acetyl-CoA was approx. 50% of the value obtained in the presence of the activator, in agreement with previous results (Cazzulo & Stoppani, 1967). In the presence of 1 mM-Mg^{2+} (excess) the double-reciprocal plot was a straight line (curve *B*). In the presence of 1 mM-ATP^{4-} (excess) the plot was strongly concave upwards (curve *D*), but the $V_{max.}$ was almost the same as for $MgATP^{2-}$ without further additions (curve *A*).

Fig. 4 shows the results presented in Fig. 2 plotted according to the Hill equation. Straight lines were always obtained, from which the apparent *n* values were calculated. The values of $V_{max.}$, $[S]_{0.5}$ and apparent *n* for $MgATP^{2-}$ under the different conditions studied are shown in Table 1.

Effect of Mn^{2+} *and* ATP^{4-} *at several* $MnATP^{2-}$ *concentrations.* Fig. 5 shows the plots of initial reaction rates against $MnATP^{2-}$ concentration. As with $MgATP^{2-}$, the increase of $MnATP^{2-}$ concentration was followed by a significant diminution of the [free ion]/[complex ion] ratio. The results in general resemble those obtained with

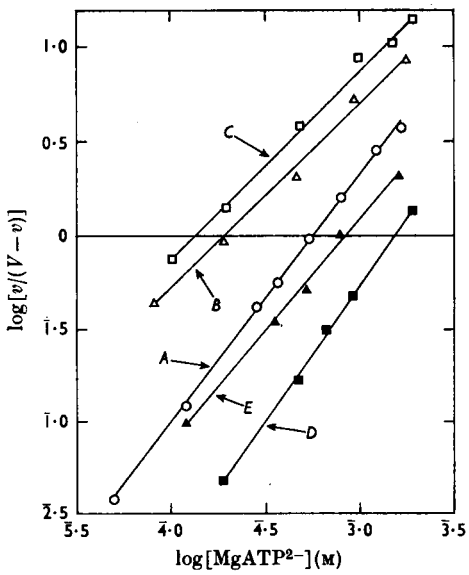

Fig. 4. Hill plots of the results presented in Fig. 2. Curves *A* (○), *B* (△), *C* (□), *D* (■) and *E* (▲) correspond to curves *A*, *B*, *C*, *D* and *E* respectively in Fig. 2.

Table 1. *$V_{max.}$, $[S]_{0.5}$ and apparent n for pyruvate carboxylase with* $MgATP^{2-}$ *as substrate*

Values were calculated from the results presented in Figs. 2–4.

Additions					
Acetyl-CoA (μM)	Mg^{2+} (mM)	ATP^{4-} (mM)	$V_{max.}$ (ΔE_{340}/min.)	$[S]_{0.5}$ (mM)	Apparent *n*
0	0	0	0·182	0·79	1·18
23	0	0	0·294	0·60	1·33
23	1·0	0	0·270	0·21	0·99
23	3·0	0	0·222	0·14	1·03
23	0	1·0	0·294	2·10	1·44

$MgATP^{2-}$, but the following differences deserve special comment. First, with 0·5 mM-Mn^{2+} (Fig. 5, curve *C*) the inhibition of the enzyme reaction was much more marked than in the similar experiment with 3 mM-Mg^{2+} (cf. Fig. 2). Secondly, in the presence of 0·5 mM-ATP^{4-} (excess) the plot (curve *D*) was strongly sigmoid. In this condition the enzyme reaction was significantly inhibited at low concentrations of $MnATP^{2-}$, whereas at the higher ones (0·1–0·4 mM-$MnATP^{2-}$) the effect was actually an activation.

Fig. 6 shows the double-reciprocal plots corresponding to the curves in Fig. 5. The plots were slightly concave upwards, either in the presence (curve *A*) or in the absence (curve *B*) of acetyl-CoA.

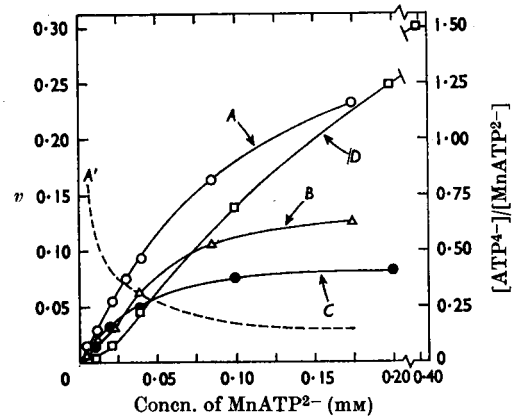

Fig. 5. Pyruvate carboxylase activity as a function of $MnATP^{2-}$ concentration. The reaction mixture contained (final concentrations): tris–HCl buffer, pH 7·0, 100 mM; $KHCO_3$, 40 mM; tris pyruvate, 8 mM; NADH, 0·44 mM; malate dehydrogenase (0·15 unit/ml.); $MnATP^{2-}$, as indicated on the abscissa; acetyl-CoA, 23 μM; pyruvate carboxylase (specific activity 18 units/mg.), 8·1 μg./ml. Curve *A* (○), without further additions; curve *B* (△), as for curve *A*, but without acetyl-CoA; curve *C* (●), with 0·5 mM-Mn^{2+} (excess); curve *D* (□), with 0·5 mM-ATP^{4-} (excess). The broken line (curve *A'*) shows the variation of the $[ATP^{4-}]/[MnATP^{2-}]$ ratio (right-hand ordinate), corresponding to the conditions for curve *A*.

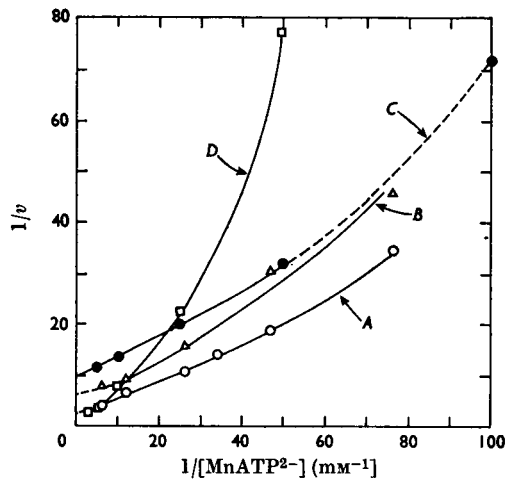

Fig. 6. Double-reciprocal plots obtained from the results presented in Fig. 5. Curves *A* (○), *B* (△), *C* (●) and *D* (□) correspond to curves *A*, *B*, *C* and *D* respectively in Fig. 5.

In the presence of acetyl-CoA addition of ATP^{4-} (curve *D*) also gave a curve concave upwards, whereas the addition of 0·5 mM-Mn^{2+} converted the plot into a straight line (curve *C*), at least for the points above 0·02 mM-$MnATP^{2-}$. The value corresponding to 0·01 mM-$MnATP^{2-}$ is less signifi-

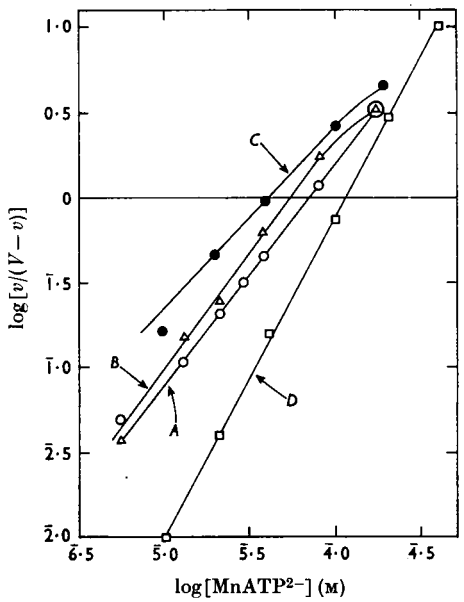

Fig. 7. Hill plots obtained from the results presented in Fig. 5. Curves *A* (○), *B* (△), *C* (●) and *D* (□) correspond to curves *A*, *B*, *C* and *D* respectively in Fig. 5.

Table 2. $V_{max.}$, $[S]_{0.5}$ *and apparent n for pyruvate carboxylase with* $MnATP^{2-}$ *as substrate*

Values were calculated from the results presented in Figs. 5–7.

Additions					
Acetyl-CoA (μM)	Mn^{2+} (mM)	ATP^{4-} (mM)	$V_{max.}$ (ΔE_{340}/min.)	$[S]_{0.5}$ (mM)	Apparent *n*
0	0	0	0·166	0·059	1·31
23	0	0	0·300	0·067	1·29
23	0·5	0	0·100	0·040	1·05
23	0	0·5	0·333	0·115	1·87

cant because of the error involved in such experimental conditions.

Fig. 7 shows the Hill plots obtained from the results presented in Fig. 5. The apparent *n* values for $MnATP^{2-}$ calculated from these data, as well as the values of $V_{max.}$ and $[S]_{0.5}$, are shown in Table 2.

Effect of several concentrations of magnesium chloride and manganese chloride at fixed $MgATP^{2-}$ *concentrations.* Fig. 8 shows the effects of different magnesium chloride and manganese chloride concentrations on enzyme activity, both at a low (0·122mM) and a nearly saturating (1·71mM) concentration of $MgATP^{2-}$. At the higher $MgATP^{2-}$

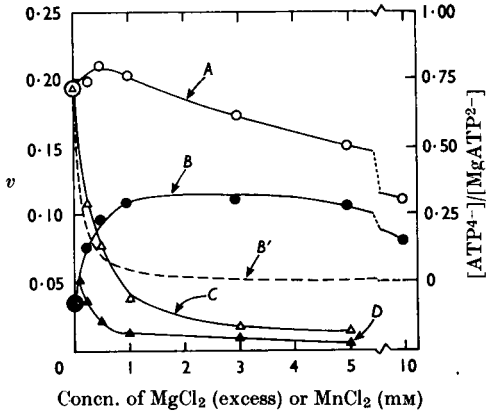

Fig. 8. Effect of $MgCl_2$ (excess) or $MnCl_2$ on pyruvate carboxylase activity. The reaction mixture contained (final concentrations): tris–HCl buffer, pH 8·0, 100mM; $KHCO_3$, 40mM; tris pyruvate, 8mM; NADH, 0·44mM; malate dehydrogenase (0·15unit/ml.); $MgCl_2$ (excess) or $MnCl_2$, as indicated on the abscissa; acetyl-CoA, 23μM; pyruvate carboxylase (specific activity 12units/mg.), 5·4μg./ml. Curve *A* (○), enzyme activity as a function of $MgCl_2$ (excess) concentration with 2mM-$MgCl_2$ (initial) and 2mM-ATP; curve *B* (●), as for curve *A* but with 0·2mM-$MgCl_2$ (initial) and 0·2mM-ATP; curve *C* (△), enzyme activity as a function of $MnCl_2$ concentration, with 2mM-$MgCl_2$ and 2mM-ATP; curve *D* (▲), as for curve *C* but with 0·2mM-$MgCl_2$ and 0·2mM-ATP. The broken line (curve *B′*) shows the variation of the $[ATP^{4-}]/[MgATP^{2-}]$ ratio (right-hand ordinate) corresponding to the conditions for curve *B*.

concentration, excess of Mg^{2+} produced its previously demonstrated inhibitory effect (Cazzulo & Stoppani, 1967), which reached 54% at 10mM-magnesium chloride (curve *A*). However, with 0·5mM-magnesium chloride there was a slight activation, probably due to the formation of complexes between remanent free ATP^{4-} with an equivalent increase of $MgATP^{2-}$ concentration. At the lower $MgATP^{2-}$ concentration (Fig. 8, curve *B*) the curve obtained was different, since the activation produced by the lower concentrations of magnesium chloride was much more significant and followed a hyperbolic curve. Moreover, the inhibitory effect of 10mM-magnesium chloride with respect to the maximal activity shown in curve *B* was much less (28%) than that observed in curve *A*. Manganese chloride proved to be a much more powerful inhibitor than magnesium chloride. In fact in the presence of 1·71mM-$MgATP^{2-}$ (Fig. 8, curve *C*) 1mM-manganese chloride caused 80% inhibition with respect to the control. At 0·122mM-$MgATP^{2-}$ (Fig. 8, curve *D*) the effect of manganese chloride was somewhat different, since with 1mM-manganese chloride the inhibition was only

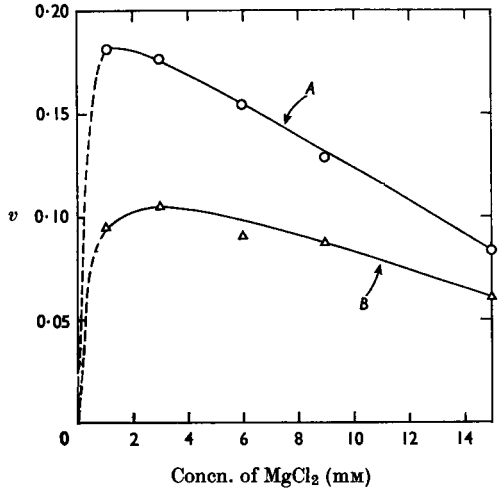

Fig. 9. Effects of $MgCl_2$ (excess) on pyruvate carboxylase activity in the presence and in the absence of acetyl-CoA. The reaction mixture contained (final concentrations): tris–HCl buffer, pH 8·0, 100 mM; $KHCO_3$, 40 mM; tris pyruvate, 8 mM; NADH, 0·44 mM; malate dehydrogenase, 0·15 unit/ml.; $MgCl_2$, as indicated on the abscissa; ATP, 0·5 mM; pyruvate carboxylase (specific activity 17 units/mg.), 5·8 μg./ml. Curve *A* (○), with 23 μM-acetyl-CoA; curve *B* (△), without acetyl-CoA.

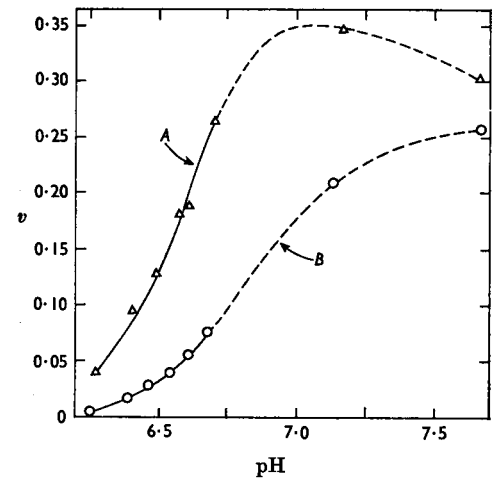

Fig. 10. Pyruvate carboxylase activity as a function of pH in the presence of $MgCl_2$ and $MnCl_2$. The reaction mixture contained (final concentrations): potassium borate–succinate buffer, 66 mM (pH values below 7·0) or tris–HCl buffer, 100 mM (pH values above 7·0); $KHCO_3$, 20 mM; KCl, 20 mM; pyruvic acid, 8 mM; NADH, 0·44 mM; malate dehydrogenase, 0·15 unit/ml.; $MgCl_2$, 12 mM; $MnCl_2$, 0·75 mM; ATP, 0·5 mM; pyruvate carboxylase (step 5 fraction; specific activity 4 units/mg.), 57 μg./ml. Curve *A* (△), with 23 μM-acetyl-CoA; curve *B* (○), without acetyl-CoA.

60% and at 0·1 mM- and 0·25 mM-manganese chloride there was actually activation.

Fig. 9 shows the effect of magnesium chloride on the enzyme activation by acetyl-CoA. In this experiment an intermediate ATP concentration (0·5 mM) was used. Excess of magnesium chloride decreased the enzyme activation by acetyl-CoA by from 90% (1 mM-total magnesium chloride) to 39% (15 mM-total magnesium chloride). Accordingly the inhibition by 15 mM-magnesium chloride, calculated on the base of the maximal activity obtained, decreased from 54% with acetyl-CoA (Fig. 9, curve *A*) to 37% when acetyl-CoA was omitted (curve *B*).

Pyruvate carboxylase activity in the presence of both magnesium chloride and manganese chloride at pH *below* 7·0. To determine the effects of magnesium chloride and manganese chloride in medium conditions nearer those reported to exist in living yeast, the enzyme activity was measured in the presence of 12 mM-magnesium chloride and 0·75 mM-manganese chloride. These cation concentrations were chosen on the base of data summarized by Eddy (1958). Fig. 10 shows the curves of enzyme activity plotted against pH in the presence (curve *A*) or in the absence (curve *B*) of acetyl-CoA. Most of the experimental determinations were performed at pH values from 6·25 to 6·65, i.e. within the physiological range of pH inside the yeast cell (Polakis & Bartley, 1965); enzyme activities at pH values above 7 are also included for comparative purposes. The curves showed, first, that the degree of activation by acetyl-CoA changed markedly with pH, being about 700% at pH 6·25 and almost negligible (9%) at pH 7·67, and, secondly, that the optimum pH values were completely different in the presence and in the absence of acetyl-CoA. The enzyme activity was critically affected by variation of pH at pH values reported to exist in living yeast.

Table 3 shows the enzyme activities obtained at pH 6·5 in the presence of magnesium chloride, manganese chloride or both together. With magnesium chloride the enzyme activity was about 15% of that obtained in the presence of manganese chloride, irrespective of the addition of acetyl-CoA. When magnesium chloride and manganese chloride were both added to the reaction mixture, the enzyme activity showed values intermediate between those obtained with each bivalent cation alone. In all cases acetyl-CoA activated the reaction about fourfold.

DISCUSSION

The activation of yeast pyruvate carboxylase by magnesium chloride and manganese chloride proved to be strongly dependent on the pH of the reaction medium. Maximal activity was at pH 8 with

Table 3. *Pyruvate carboxylase activity in the presence of magnesium chloride or manganese chloride or both at* pH 6·5

The reaction mixture was as described in the legend of Fig. 10, except that 66 mM-potassium borate–succinate buffer, pH 6·5, was used and acetyl-CoA, $MgCl_2$ and $MnCl_2$ were added as indicated.

Addition of acetyl-CoA (μM)	Further addition(s)	Activity (ΔE_{340}/min.) $MgCl_2$ (12 mM)	$MnCl_2$ (0·75 mM)	$MgCl_2$ (12 mM) + $MnCl_2$ (0·75 mM)
0		0·015	0·110	0·036
23		0·060	0·400	0·132

$MgATP^{2-}$ and at pH 7 with $MnATP^{2-}$; the latter was about 40% of the maximal activity with $MgATP^{2-}$. At the respective pH optima the plots of the initial reaction velocities against $MgATP^{2-}$ (or $MnATP^{2-}$) concentrations showed a slightly sigmoid pattern (Figs. 2 and 5). This sigmoidicity was not evident in a previous study (Cazzulo & Stoppani, 1967) because of the higher $MgATP^{2-}$ concentrations employed. The sigmoid shape of the curves may reflect the variation of the relative concentration of ATP^{4-} (an inhibitor of the enzyme reaction) with respect to the concentration of the substrate ions $MgATP^{2-}$ or $MnATP^{2-}$ (Figs. 2 and 5 respectively). Thus, when the relative concentration of ATP^{4-} decreased [at the higher $MgATP^{2-}$ (or $MnATP^{2-}$) concentrations], the inhibitory effect of ATP^{4-} would become negligible and a relatively higher reaction velocity should be expected, as actually occurred. The more marked sigmoidicity of the curves obtained in the presence of ATP^{4-} in excess (Figs. 2 and 5) leads to the assumption that ATP^{4-} may act as a negative allosteric modifier, and this latter effect may also contribute to the peculiar kinetics of the enzyme activation by $MgATP^{2-}$ (Fig. 2) and $MnATP^{2-}$ (Fig. 5). The results obtained might also be explained by a homotropic co-operative effect of $MgATP^{2-}$ (or $MnATP^{2-}$), as has been postulated for the sheep kidney pyruvate carboxylase acting with $MgATP^{2-}$ as substrate (Keech & Barritt, 1967). However, this would require that all magnesium chloride (or manganese chloride) and ATP should be as the respective complex ions, which in our experimental conditions did not occur.

The effect of Mg^{2+} on the enzyme kinetics with respect to the $MgATP^{2-}$ concentration (Fig. 2) would depend primarily on the variation of ATP^{4-} concentration. In fact, an excess of cation converts most of the ATP^{4-} into $MgATP^{2-}$, increasing the concentration of the latter complex and decreasing that of the inhibitory ATP^{4-} ion. Both effects are particularly significant at the lower magnesium chloride–ATP concentrations, which fits in with the conversion of the sigmoid curve into hyperbolae, as shown in Fig. 2. Accordingly, the $[S]_{0.5}$ values for the $MgATP^{2-}$ complex decreased on the addition of Mg^{2+} (Table 1). The possibility that Mg^{2+} is an activator of yeast pyruvate carboxylase, as for the sheep kidney enzyme (Keech & Barritt, 1967), seems unlikely, since, unlike what occurred with the latter enzyme, Mg^{2+} actually decreased the $V_{max.}$ of the reaction (Table 1) and, at minimal ATP^{4-} concentrations, Mg^{2+} above 1 mM (excess) inhibited the reaction (Fig. 8, curve *A*).

The different effects observed with free Mg^{2+} or Mn^{2+} in Figs. 2 and 5 can be explained by the different inhibitory power of these cations as compared with free ATP^{4-}. In fact, in the presence of $MgATP^{2-}$ (Fig. 2) ATP^{4-} was a much stronger inhibitor than Mg^{2+} and therefore, by forming a complex with ATP^{4-}, excess of Mg^{2+} would de-inhibit the enzyme. On the other hand, Mn^{2+} proved to be an enzyme inhibitor stronger than ATP^{4-}, as shown in Table 2, where Mn^{2+} decreased $V_{max.}$ to 30% of the control value. However, excess of Mn^{2+} would convert free ATP^{4-} into $MnATP^{2-}$ and thus produce a small enzyme activation. This is shown in Fig. 5 by the conversion of the sigmoid curve (curve *A*) into a hyperbola (curve *C*).

The curves obtained at extremely low concentrations of free ATP^{4-}, either in the presence of $MgATP^{2-}$ (Fig. 2, curves *B* and *C*) or $MnATP^{2-}$ (Fig. 5, curve *C*), were hyperbolae, which became sigmoid when ATP^{4-} concentration increased. This would fit in with the already postulated allosteric inhibition of yeast pyruvate carboxylase by ATP^{4-}. In the presence of ATP^{4-} (excess) $V_{max.}$ was scarcely affected and $[S]_{0.5}$ increased significantly (Tables 1 and 2), which are consistent with the competitive type of inhibition.

Yeast pyruvate carboxylase seems to act more efficiently with $MnATP^{2-}$ than with $MgATP^{2-}$ even at pH 8, provided that the concentration of ATP is low. In fact, low manganese chloride concentrations activated the enzyme in the presence of 0·122 mM-$MgATP^{2-}$ (Fig. 8), and at the respective pH optima the enzyme affinity for $MnATP^{2-}$ was greater than

for $MgATP^{2-}$ as shown by the $[S]_{0.5}$ values, namely 0·067mM (for $MnATP^{2-}$) and 0·60mM (for $MgATP^{2-}$) (Tables 1 and 2).

To evaluate the possible role of Mg^{2+} and Mn^{2+} as physiological activators of pyruvate carboxylase, the experiments described in Fig. 10 and Table 3 were performed in medium conditions similar to those existing inside the yeast cell, namely pH 6·25–6·65 (Polakis & Bartley, 1965), ATP concentration 0·3–0·7mM (Polakis & Bartley, 1966), magnesium chloride concentration not less than 20mM and manganese chloride concentration 0·7mM (cation values calculated from data quoted by Eddy, 1958). The results obtained as well as those presented in Fig. 1 support the idea that $MnATP^{2-}$ is the more physiological substrate for the yeast pyruvate carboxylase; at pH values below 7·0 the enzyme activity was higher with $MnATP^{2-}$ (Fig. 1), and at pH 6·5 manganese chloride was a more effective activator than magnesium chloride, even in the presence of a great excess (16-fold) of magnesium chloride (Table 3). Further, the plot of reaction velocity against pH in the presence of both cations and acetyl-CoA (Fig. 10, curve *A*) resembled closely the one obtained for $MnATP^{2-}$ in the presence of acetyl-CoA (Fig. 1, curve *C*).

The inhibition by free ATP^{4-} is unlikely to be physiological, since the excess of Mg^{2+} and Mn^{2+} inside the yeast cell would keep the concentration of free ATP^{4-} at values that do not affect the enzyme activity. On the other hand, other adenosine phosphates, such as ADP, might be more significant, and these are dealt with in the accompanying paper (Cazzulo & Stoppani, 1969).

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