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Properties and Function of Yeast Pyruvate Carboxylase*

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Cannata and Stoppani (1, 2) found that bakers' yeast contains an adenosine diphosphate-dependent phosphoenolpyruvate carboxykinase, and they ascribed the essential physiological role of oxaloacetate synthesis to it. Doubts concerning their interpretation arose, however, when it was revealed (3, 4) that cell-free preparations of the same microorganism could catalyze the direct carboxylation of pyruvate to oxaloacetate in the presence of adenosine triphosphate. Yeast pyruvate carboxylase was shown to be inhibited by oxalate and stimulated by either coenzyme A or its converted form, acetyl coenzyme A. Thus, yeast pyruvate carboxylase differs from the avian enzyme (5, 6), which depends absolutely on the presence of acetyl coenzyme A, and from the *Aspergillus niger* (7) and *Pseudomonas citronellolis* (8) enzymes, which do not have any requirement for this compound. The present paper describes a procedure for purification of pyruvate carboxylase from bakers' yeast, and some of the properties of this enzyme, including its lability, affinity for various substrates, activators, and inhibitors, and the effect of pH.

Attempts by Cannata and Stoppani (1) to demonstrate a malate enzyme in *Saccharomyces cerevisiae* have been unsuccessful. We have now found, however, as will be reported in this article, that the formation of this enzyme in another yeast, *Rhodotorula glutinis*, depends on the presence of malic acid or other related compounds in the growth medium. Blanchard, Korkes, Campillo, and Ochoa (9) have previously reported that an extremely active malate enzyme is present in *Lactobacillus arabinosus* after culturing it in the presence of malic acid. Some of the experiments included in the present communication have been carried out in order to study the function of pyruvate carboxylase and related enzymes in both glycolysis and gluconeogenesis in yeast. For this purpose, two species have been grown on different carbon sources, and the specific activities of each enzyme have been estimated in the corresponding crude extracts. The results showed that pyruvate carboxylase varied only slightly, whereas pyruvate kinase, phosphoenolpyruvate carboxykinase, and malate enzyme drastically changed according to the carbon compound consumed by the yeast. The data obtained shed new light on the significance of these enzymes in several outstanding metabolic pathways.

EXPERIMENTAL PROCEDURE

Yeast Species and Growth—*S. cerevisiae* (bakers' yeast) was acquired from the market. *R. glutinis* (strain RH-1413) and *Hansenula anomala* (strain W-10) were kindly supplied by the Departamento de Fermentaciones Industriales, C.S.I.C., Madrid, Spain. They were grown aerobically at 30° with vigorous shaking in the synthetic medium of Olson and Johnson (10), but with one of the following compounds as the only carbon source: glucose, 2%; pyruvate, 4%; malate, 3%; aspartate, 0.5%; acetate, 6%. During the logarithmic phase, the cells were harvested by slow speed centrifugation and washed with 0.05 M Tris-HCl, pH 7.6.

Preparation of Cell-free Extracts—The yeast crude extracts were prepared in the cold by grinding fresh cells in a mortar with twice their weight of alumina and extracted with 0.05 M Tris-HCl, pH 7.6. After centrifugation for 15 min at 15,000 × *g*, the supernatant layer was used as such for the enzyme assays.

Measurement of Enzymes—Pyruvate carboxylase was assayed by a modification of the procedure of Utter and Keech (5). Pyruvate kinase (11) and malate enzyme (12) were determined by conventional optical methods. Phosphoenolpyruvate carboxykinase was assayed by measuring $^{14}CO_2$ fixation under essentially the conditions described by Cannata and Stoppani (1), except that lactate dehydrogenase and NADH were added to avoid interference by pyruvate carboxylase (4). Enzyme units are expressed as micromoles of substrate utilized or product formed per min.

Analytical Methods—NADH oxidation and NADP reduction were determined at room temperature by measuring the change in absorbance at 340 mμ in cuvettes with a light path of 1 cm. $^{14}CO_2$ fixation was estimated by measuring the radioactive carbon in stainless steel planchets with either a gas flow or a thin window counter with efficiencies of approximately 20 and 5%, respectively. Protein was determined by the methods of Lowry *et al.* (13) and Warburg and Christian (14).

Materials—Lactate dehydrogenase and phosphotransacetylase were acquired from Boehringer und Soehne. Malate dehydrogenase, glutamate-oxaloacetate transaminase, and pyruvate kinase were supplied by Sigma Chemical Company.

ATP, ADP, acetylphosphate, biotin, CoA, DEAE-cellulose, NADH, NADP, phosphoenolpyruvate, and protamine sulfate were purchased from Sigma Chemical Company. Avidin and Sephadex G-25 were obtained from Nutritional Biochemicals Corporation and Pharmacia, respectively. Alumina (A-305) was a gift from Alcoa Company. $^{14}CO_2$ was acquired from The Radiochemical Centre. Acetyl-CoA was prepared as described by Stadtman (15). All of the chemicals used were of analytical grade.

RESULTS

Preparation of Pyruvate Carboxylase—The enzyme has been purified about 140-fold with the crude extract from bakers'

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yeast, prepared as described under "Experimental Procedure." The purification procedure summarized in Table I was carried out as follows. To the crude extract (Fraction I) containing 20 mg of protein per ml, a 2% solution of protamine sulfate was added in the proportion of 0.1 mg of protamine sulfate per mg of protein. After standing for 10 min, the suspension was centrifuged at 27,000 × *g* for 10 min, and the sediment was discarded. The resulting supernatant (Fraction II) was then treated with the same volume of 90% ammonium sulfate to bring the solution to a final concentration of 45% saturation. The suspension was allowed to stand for 20 min and centrifuged at 27,000 × *g* for 10 min. The protein precipitate (Fraction III) contained all of the activity of the original extract and it was quite stable when kept in a deep freeze for at least 2 weeks. The precipitate was dissolved in 0.05 M Tris-HCl, pH 7.6 (in the proportion of 2 ml of buffer per 20 ml of the starting crude extract), and each 2-ml portion was passed through a Sephadex G-25 column (10 cm high and 2 cm in diameter) in order to remove the ammonium sulfate that was present. The passing solution was diluted up to 10 ml with 0.05 M Tris-HCl, pH 7.6, and ammonium sulfate was added to give a fixed final concentration of 0.02 M to protect the enzyme during the next purification step. This solution (Fraction IV) was then passed through a DEAE-cellulose column (6 cm high and 1 cm in diameter) which was previously treated as described by Seubert and Remberger (8) and equilibrated with 0.05 M Tris-HCl (pH 7.6)-0.02 M ammonium sulfate. To get rid of unwanted protein, the column was washed with 20 ml of 0.1 M Tris-HCl (pH 7.6)-0.02 M ammonium sulfate. Pyruvate carboxylase was finally eluted with 5 ml of 0.15 M Tris-HCl (pH 7.6)-0.02 M ammonium sulfate (Fraction V), and used as such for the study of the properties of the enzyme described below.

TABLE I

Purification of bakers' yeast pyruvate carboxylase

Enzyme assays were carried out by measuring $^{14}CO_2$ fixation as follows. A total volume of 1.0 ml contained, in addition to the pyruvate carboxylase preparation, 100 μmoles of Tris-HCl (pH 7.6), 10 μmoles of sodium pyruvate, 15 μmoles of $KH^{14}CO_3$ (6 μC), 10 μmoles of ATP, 10 μmoles of $MgCl_2$, 0.3 μmole of CoA, 10 μmoles of cysteine, 4 units of glutamate-oxaloacetate transaminase, and 15 μmoles of sodium glutamate. After incubation for 30 min at 30° and deproteinization with 0.1 ml of 50% trichloroacetic acid, the mixture was centrifuged and the radioactivity was determined in an aliquot of the supernatant fraction with an end window counter as described under "Experimental Procedure."

Fraction	Total protein	Total activity	Recovery	Specific activity
	mg	*milliunits*	%	*milliunits/mg*
I. Crude extract	400	640	100	1.6
II. Protamine sulfate supernatant	88	616	96	7.0
III. Ammonium sulfate precipitate	11.6	661	103	57.0
IV. Sephadex G-25 filtrate	9.0	495	77	55.0
V. DEAE-cellulose eluate	0.37	77	12	207.0

Pyruvate Carboxylase Assays—During the purification stages, pyruvate carboxylase was assayed as described in Table I by

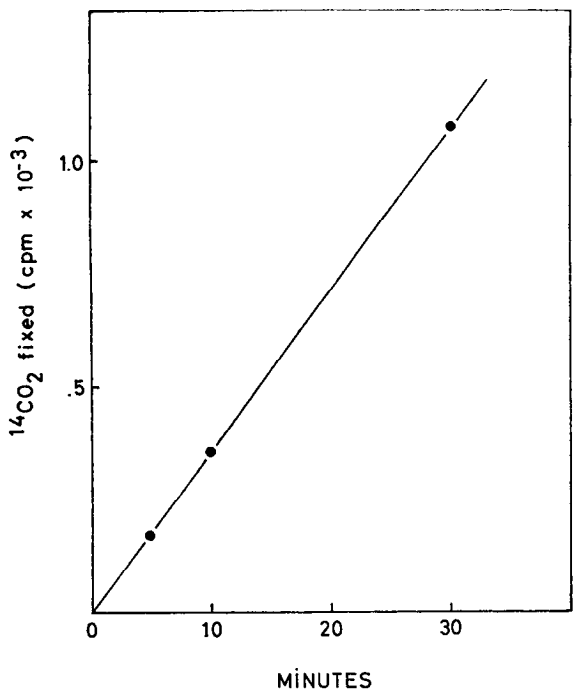


FIG. 1 (*left*). Proportionality of $^{14}CO_2$ fixation of pyruvate carboxylase with incubation time. Experimental conditions were as in the standard assay described in Table I. Pyruvate carboxylase (Fraction V), 22 μg.

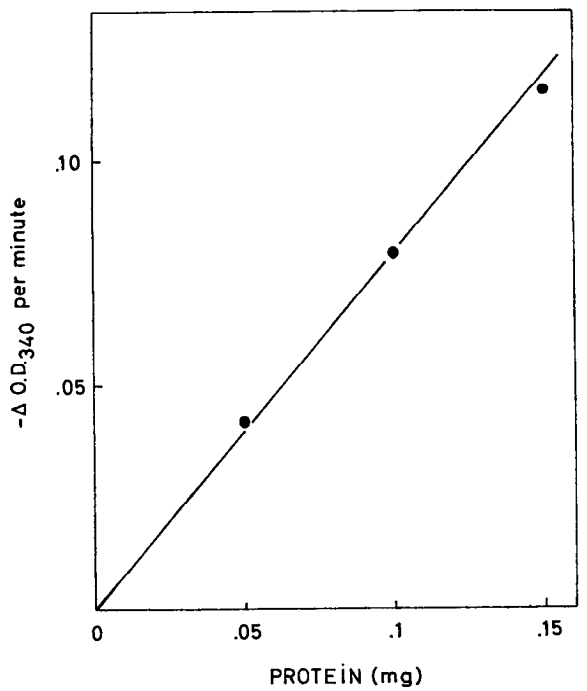


FIG. 2 (*right*). Proportionality of pyruvate carboxylase activity with protein added. The assays were carried out spectrophotometrically. Each cuvette contained in a final volume of 3 ml: 275 μmoles of Tris-HCl (pH 7.6), 20 μmoles of sodium pyruvate, 30 μmoles of $NaHCO_3$, 10 μmoles of ATP, 20 μmoles of $MgCl_2$, 0.9 μmole of CoA, 20 μmoles of cysteine, 0.4 μmole of NADH, 0.12 unit of malate dehydrogenase, and the indicated amounts of pyruvate carboxylase (Fraction V). The optical density changes were measured as described under "Experimental Procedure."

measuring $^{14}CO_2$ fixation in the presence of glutamate and glutamate-oxaloacetate transaminase (4). The proportionality of $^{14}CO_2$ fixation with the incubation time is shown in Fig. 1. Pyruvate carboxylase was alternatively assayed spectrophotometrically by measuring oxaloacetate formation with the aid of malate dehydrogenase and NADH (4). A typical spectrophotometric assay showing the effect of the amount of enzyme on the reaction rate is shown in Fig. 2.

Lability of Pyruvate Carboxylase—The purified enzyme was quite unstable and it lost about 25% of its activity when kept for 3 hours at 22°, the inactivation being greater at 0°. After a period of 24 hours at either temperature, more than 80% of the original activity had disappeared (*cf.* Reference 16). As shown by Utter, Keech, and Scrutton (16) for the avian enzyme, either sucrose (1 M) or ammonium sulfate (45% saturation) highly stabilized the yeast pyruvate carboxylase.

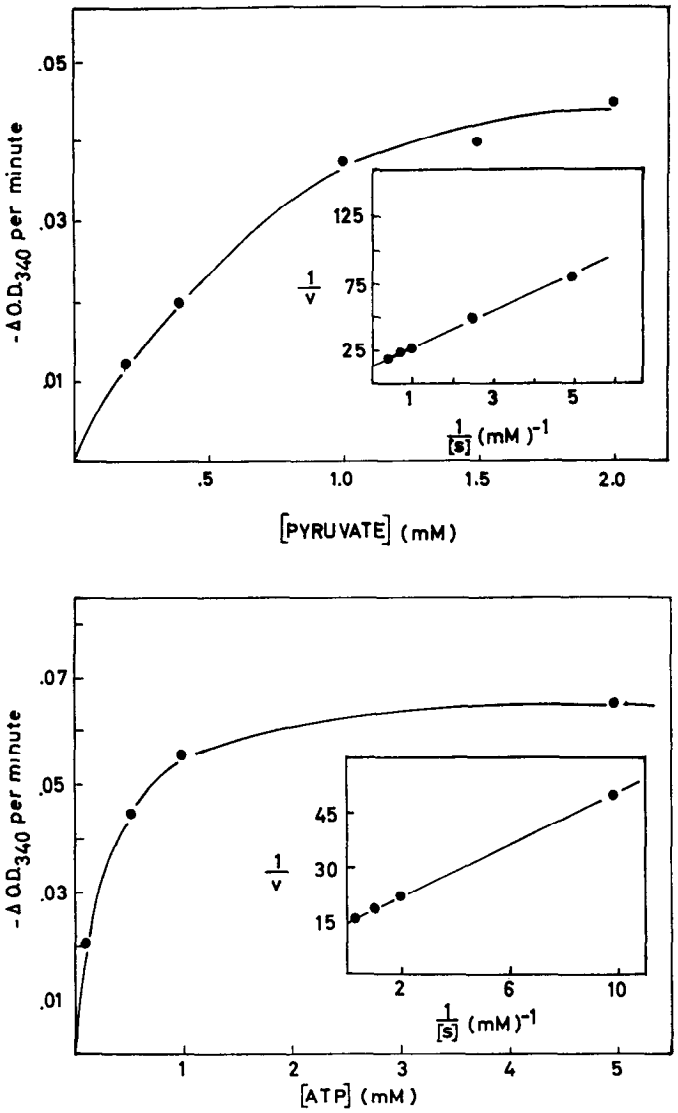


FIG. 3. Effect of pyruvate and ATP concentration on pyruvate carboxylase activity. The assays were carried out spectrophotometrically. Each cuvette contained in a final volume of 3 ml: 74 μg of pyruvate carboxylase (Fraction V), 0.24 unit of malate dehydrogenase, 500 μmoles of Tris-HCl (pH 8.4), 30 μmoles of $KHCO_3$, 20 μmoles of $MgCl_2$, 0.9 μmole of CoA, 20 μmoles of cysteine, 0.4 μmole of NADH, and either 10 μmoles of ATP and pyruvate as indicated or 20 μmoles of pyruvate and ATP as indicated. In the second case, the reaction mixture also contained an ATP-regenerating system composed of 0.2 unit of pyruvate kinase, 3 μmoles of phosphoenolpyruvate, and 100 μmoles of KCl. The optical density changes were measured as described under "Experimental Procedure."

TABLE II

Effect of bicarbonate concentration on pyruvate carboxylase activity

Experimental conditions were as in Fig. 3, except that 20 μmoles of pyruvate were used and $KHCO_3$ was added as indicated. All of the cuvettes were equalized in K^+ to the maximal concentration of $KHCO_3$ by adding KCl (*cf.* Fig. 4).

HCO_3^- added	Reaction rate	Calculated K_m
mM	*ΔO.D.340/min*	*mM*
Experiment 1		
None	0.040	
10.0	0.110	3.0
33.3	0.140	
Experiment 2		
None	0.065	
6.7	0.240	2.4
33.3	0.320	
Experiment 3		
None	0.045	
3.3	0.140	2.8
33.3	0.280	

Effect of pH on Pyruvate Carboxylase Activity—Under the experimental conditions described in Table I, the enzyme exhibited maximal activity at pH 8.3.

K_m Values for Reaction Components—As shown previously (4) with the crude extract, the formation of oxaloacetate from pyruvate and CO_2 catalyzed by the purified enzyme, required ATP and the presence of Mg^{++}. The effect of pyruvate concentration on pyruvate carboxylase activity is presented in Fig. 3. By plotting the reciprocal of the pyruvate concentration against the reciprocal of the reaction velocity, a Michaelis constant of 0.80 mM was found for this substrate. Fig. 3 also shows the effect of ATP concentration on pyruvate carboxylase activity. Since the enzyme preparation was not completely free of ATPase activity, an ATP-regenerating system composed of pyruvate kinase and phosphoenolpyruvate was used in these experiments. From the data seen in the Lineweaver-Burk plot (Fig. 3), a K_m of 0.24 mM for ATP was calculated.

In order to measure the K_m value for bicarbonate, it was necessary to take into account the endogenous concentration of this compound in the reaction mixture since it could not be removed at the pH used by vacuum and gassing procedures. After determining the reaction rate when no bicarbonate was added and the V_{max} at saturation of this substrate, the K_m value could be calculated from any concentration of bicarbonate added and from the corresponding velocity of the reaction by applying the Michaelis-Menten equation. From the data of the three experiments presented in Table II, an average K_m value for bicarbonate of 2.7 mM was estimated. From these data, it can also be calculated that, under the experimental conditions used, the endogenous bicarbonate concentration of the reaction mixture varied between 0.5 and 1.1 mM. No assumptions concerning the active species of CO_2 have been made since there is no evidence at present on this point (6).

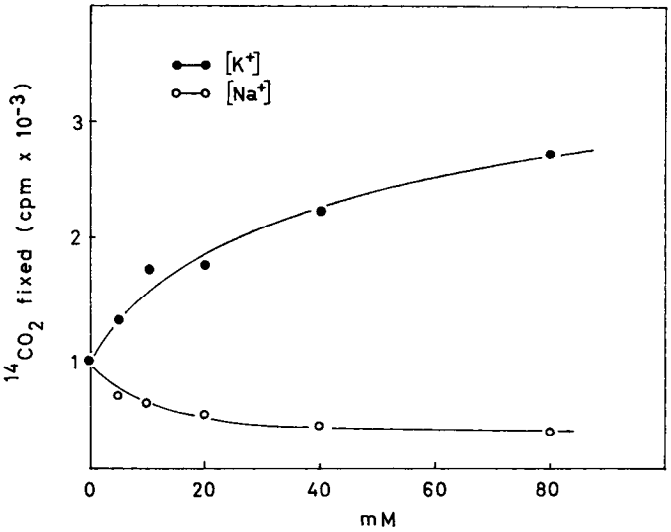
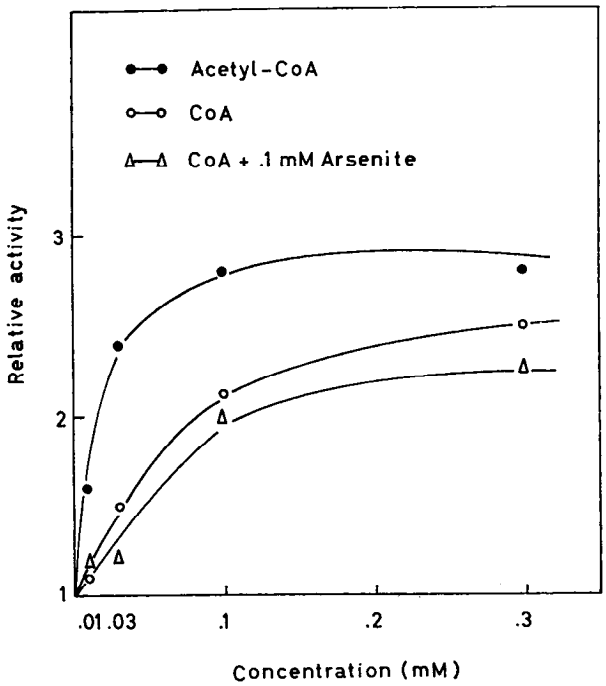


FIG. 4. Effect of K^+ and Na^+ concentrations on pyruvate carboxylase activity. The assays were carried out by measuring $^{14}CO_2$ fixation. The reaction mixture contained in a final volume of 1 ml: 18 μg of pyruvate carboxylase (Fraction V), 2 units of glutamate-oxaloacetate transaminase freed of NH_4^+ by treatment with Sephadex G-25, and the following in micromoles: Tris-HCl (pH 8.4), 100; pyruvic acid, 5; $KH^{14}CO_3$, 0.9 (3 μC); ATP (acid form), 2.5; $MgCl_2$, 10; CoA, 0.3; cysteine, 10; glutamic acid, 10; KCl or NaCl as indicated. Incubation time was 3 min. Other experimental conditions were as described in Table I, except that the radioactivity was determined with a gas flow counter.

In measuring the K_m value for Mg^{++}, precautions were taken to avoid complications which might arise owing to its chelation with the ATP present in the reaction mixture. Therefore, the ATP concentration was reduced to 1 mM and the Mg^{++} concentrations used were above this level (*cf.* Reference 6). Under these conditions, a K_m value for Mg^{++} of 4.2 mM was calculated.

Effect of K^+ and Na^+ on Pyruvate Carboxylase Activity—Bloom and Johnson (7) have reported stimulation of *Aspergillus* pyruvate carboxylase by K^+. In order to study the effect of this ion on the yeast enzyme, the commercial alkali salts of the reaction components were converted to their acid forms, by passage through a small Dowex 50-W column, and the concentration of $KH^{14}CO_3$ in the reaction mixture was greatly reduced. As can be seen in Fig. 4, K^+ stimulated $^{14}CO_2$ fixation increasingly with concentration whereas Na^+ inhibited it.

Activation of Pyruvate Carboxylase by CoA and Acetyl-CoA—The previous finding (4) that the pyruvate carboxylase activity was increased about 2-fold by CoA has now been reinvestigated in more detail with the purified enzyme. Since the possibility could not be excluded that, even with the purified preparation, acetyl-CoA could be formed from pyruvate and CoA in our system, arsenite was added to the reaction mixture at concentrations up to 10 times higher than those reported to inhibit the pyruvate oxidation system (17, 18). It was found that from 0.1 mM to 0.3 mM arsenite had little effect on the carboxylase reaction, either in the presence or in the absence of CoA and acetyl-CoA. Fig. 5 shows the effect of increasing concentrations



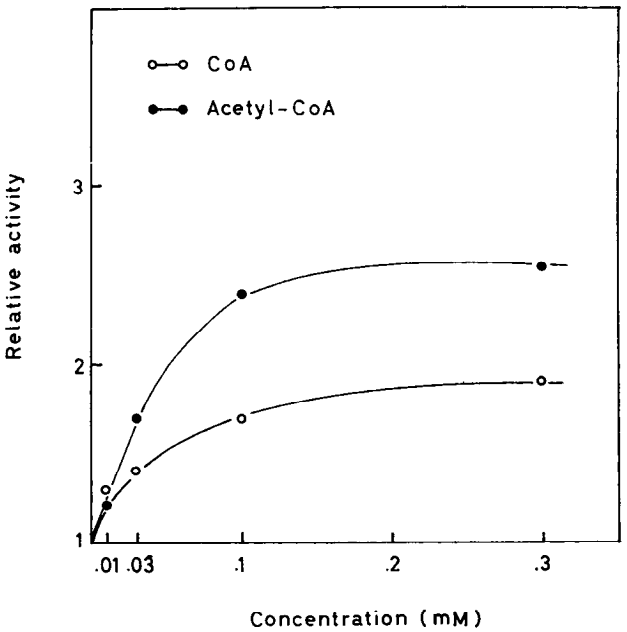


FIG. 5 (*left*). Effect of CoA and acetyl-CoA on pyruvate carboxylase activity. Assays were carried out by measuring $^{14}CO_2$ fixation. The reaction mixture contained in a final volume of 1.1 ml: pyruvate carboxylase (Fraction V), 7 μg; glutamate-oxaloacetate transaminase, 2 units; Tris-HCl (pH 8.4), 100 μmoles; sodium pyruvate, 10 μmoles; $KH^{14}CO_3$, 5 μmoles (8 μC); ATP, 5 μmoles; $MgCl_2$, 10 μmoles; KCl, 21 μmoles; sodium glutamate, 15 μmoles; and CoA, acetyl-CoA, and potassium arsenite as indicated. Incubation time was 15 min. Other experimental conditions are described in Table I.

FIG. 6 (*right*). Effect of CoA and acetyl-CoA enzymatically formed from acetyl-CoA and CoA, respectively, on pyruvate carboxylase activity. Assays were carried out by measuring $^{14}CO_2$ fixation. The reaction mixture contained in a final volume of 1.15 ml: pyruvate carboxylase, (Fraction V), 7 μg; glutamate-oxaloacetate transaminase, 2 units; Tris-HCl (pH 8.4), 100 μmoles; sodium pyruvate, 10 μmoles; $KH^{14}CO_3$, 5 μmoles (8 μC); ATP, 5 μmoles; $MgCl_2$, 10 μmoles; KCl, 90 μmoles; sodium glutamate, 15 μmoles; one of the two following enzyme systems: O——O, acetyl-CoA (as indicated), potassium arsenate (45 μmoles), and phosphotransacetylase (4 units) for generating CoA from acetyl-CoA; or ●——●, CoA (as indicated), acetylphosphate (5 μmoles), and phosphotransacetylase (4 units) for generating acetyl-CoA from CoA. Other experimental conditions were as described in Table I.

of acetyl-CoA and CoA (with and without arsenite) on pyruvate carboxylase activity, and it demonstrates that, although acetyl-CoA is a more effective activator, both compounds produce comparable effects. In order to prove unequivocally that these conclusions were correct, experimental conditions were devised that could ensure the steady maintenance of the level of CoA and acetyl-CoA even at low concentrations. For this purpose, the two generating enzyme systems of CoA and acetyl-CoA described in Fig. 6 were employed. In complete agreement with the results of Fig. 5, Fig. 6 shows that activation is lower when added acetyl-CoA is converted into CoA than when added CoA is converted into acetyl-CoA. The ratios of basal activity to acetyl-CoA- and CoA-stimulated activity were relatively constant with different preparations.

Effect of Inhibitors—The inhibition by oxalate of pyruvate carboxylase activity previously reported (3, 4) has now been re-examined with the purified enzyme at different concentrations of the reaction components. It is apparent from the data presented in Table III that the inhibitory effect of oxalate was independent of the concentrations of pyruvate, bicarbonate, and ATP and thus corresponded to the noncompetitive type. In the case of Mg^{++}, a stronger inhibition was observed when the concentration of this cation was reduced to 1 mM and kept 5 times lower than that of ATP. Owing to the chelation of the Mg^{++} with the ATP, the real concentration of free Mg^{++} in the reaction mixture is even lower, and the superimposed inhibition observed under these conditions can be explained by an additional removal of Mg^{++} by oxalate. For calculating the enzyme-inhibitor constant, much higher amounts of Mg^{++} (40 μmoles) than of ATP (10 μmoles) were employed, and the experiments were carried out at different concentrations of pyruvate in the presence and absence of oxalate (0.1 mM) as indicated in Fig. 7. Under these conditions, a K_i value for oxalate of 0.07 mM was calculated from the plots of reciprocal velocity against reciprocal substrate concentration.

TABLE III

Inhibition by oxalate of pyruvate carboxylase activity at different concentrations of reaction components

The assays were carried out by measuring $^{14}CO_2$ fixation. The complete system included in a final volume of 1 ml: 7 μg of pyruvate carboxylase (Fraction V), 100 μmoles of Tris-HCl (pH 8.4), 5 μmoles of sodium pyruvate, 10 μmoles (4 μC) of $KH^{14}CO_3$, 5 μmoles of ATP, 10 μmoles of $MgCl_2$, 0.3 μmole of CoA, 2 units of glutamate-oxaloacetate transaminase, and 15 μmoles of sodium glutamate. Other experimental conditions were as described in Table I, except that the radioactivity was determined with a gas flow counter.

System	$^{14}CO_2$ fixed		Inhibition
	No oxalate	Oxalate (0.1 mM)	
	cpm × 10^{-3}		%
1. Complete	47.5	26.9	54
2. Complete but 1 mM Mg^{++}	4.8	0.5	91
3. Complete but 0.5 mM ATP	25.9	12.5	52
4. Complete but 0.5 mM pyruvate	22.0	9.5	54
5. Complete but 1 mM HCO_3^-	12.5	5.3	58

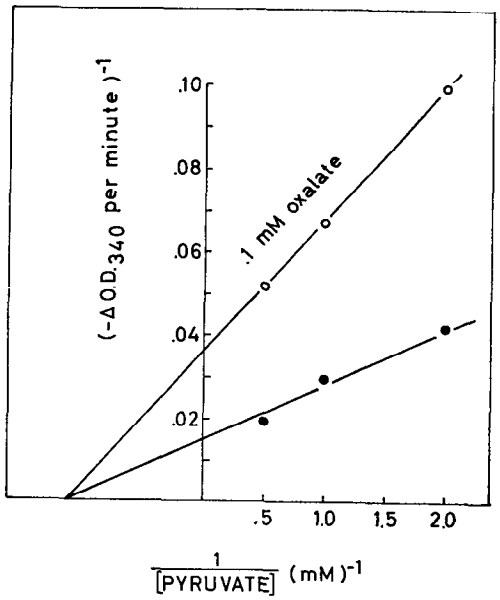


FIG. 7. Noncompetitive inhibition of pyruvate carboxylase by oxalate. The assays were carried out spectrophotometrically. Each cuvette contained in a final volume of 3 ml: pyruvate carboxylase (Fraction V), 74 μg; Tris-HCl (pH 8.4), 500 μmoles; $KHCO_3$, 150 μmoles; ATP, 10 μmoles; $MgCl_2$, 40 μmoles; malate dehydrogenase, 0.24 unit; NADH, 0.4 μmole; sodium pyruvate and potassium oxalate as indicated. The optical density changes were measured as described under "Experimental Procedure."

TABLE IV

Effect of avidin and biotin on pyruvate carboxylase

The assays were carried out by measuring $^{14}CO_2$ fixation as described for System 1 in Table III except for the avidin and biotin additions. Biotin was incubated with avidin for 5 min before mixing with the enzyme system.

Addition	^{14}CO fixed
	cpm × 10^{-3}
None	6.3
62.5 milliunits of avidin	2.1
125 milliunits of avidin	0.7
62.5 milliunits of avidin + 0.5 μmole of biotin	6.5
125 milliunits of avidin + 0.5 μmole of biotin	6.5
0.5 μmole of biotin	6.9

The almost complete inhibition of the $^{14}CO_2$-fixing reaction by avidin (*cf.* Reference 19) and the prevention of this inhibitory effect by preincubating the avidin with biotin (Table IV) demonstrated that yeast pyruvate carboxylase, like those of liver (16), *Pseudomonas* (8), and *Aspergillus* (7), is a biotin enzyme.

Levels of Pyruvate-related Enzymes in Yeasts Grown with Different Carbon Sources—Table V summarizes the activities of pyruvate carboxylase, pyruvate kinase, phosphoenolpyruvate carboxykinase, and malate enzyme in two yeast species grown as outlined under "Experimental Procedure" in media containing either glucose, pyruvate, malate, aspartate, or acetate as the only carbon source. The specific activity of each enzyme was determined in the corresponding crude extracts as described above, pyruvate carboxylase being assayed radiochemically. It can be seen from the data presented in this table that pyruvate

TABLE V

Activities of pyruvate-related enzymes in yeasts grown on different carbon sources

Yeast and carbon source	Specific activity			
	Pyruvate carboxylase	Pyruvate kinase	Phosphoenolpyruvate carboxykinase	Malate enzyme
	milliunits/mg protein			
Rhodotorula glutinis				
Glucose	14.2		1.3	<0.7
	44.0	167	1.3	
Pyruvate	18.3	4.3	5.2	8.7
	18.3	1.8	5.0	10.3
Malate	5.8	1.7	18.3	78.4
	5.0	9.3	14.3	36.6
	12.5	2.7	21.6	26.5
Aspartate	7.7	2.3	15.7	63.6
	6.7	1.5	14.9	56.6
	11.6	8.1	29.6	17.0
Acetate	12.2	<0.7	6.6	
	10.3	1.2	7.0	43.2
Hansenula anomala				
Glucose	20.3	358	0.1	<0.7
Acetate	9.3	1.25	8.2	<0.7
	12.0	<0.7	22.4	<0.7

carboxylase did not show great variations in response to the carbon substrate used by the cells. By contrast, pyruvate kinase, phosphoenolpyruvate carboxykinase, and malate enzyme changed drastically according to the carbon compound consumed by the cells. The level of pyruvate kinase was very high only when glycolysis was active, *i.e.* in cells grown on glucose. Phosphoenolpyruvate carboxykinase was also found to vary greatly but in the opposite way, being much higher when gluconeogenesis was operative; thus, it was low in the presence of glucose as substrate and increased largely with any of the other carbon sources. Finally, after culture of the yeasts in the presence of any one of the other substrates, the NADP-dependent malate enzyme, which was always undetectable in cells grown on glucose, appeared abundantly in *Rhodotorula* but not in *Hansenula*. The conclusions that can be drawn from these results will be considered in some detail and compared with those of other authors in the following section.

DISCUSSION

The pyruvate carboxylase described here is apparently very similar to those previously found in avian liver (5, 6, 16), *P. citronellolis* (8), and *A. niger* (7). An especially interesting property of the yeast carboxylase is its activation by both acetyl-CoA (*cf.* Reference 19) and CoA, since the chicken liver enzyme depends absolutely on acetyl-CoA but not on CoA and those of *Pseudomonas* and *Aspergillus* do not require acetyl-CoA. The brief report by Fuller *et al.* (20) showing that the ATP-dependent carboxylation of pyruvate in the photosynthetic bacterium *Chromatium* (21) was stimulated by acetyl-CoA suggests that the bacterial carboxylase may be analogous to the yeast enzyme from this point of view. The strong noncompetitive inhibition of yeast pyruvate carboxylase by oxalate (K_i = 0.07 mM) may imply a feedback control mechanism since oxalate has been shown to be the product of oxaloacetate metabolism in *A. niger* under certain cultural conditions (22).

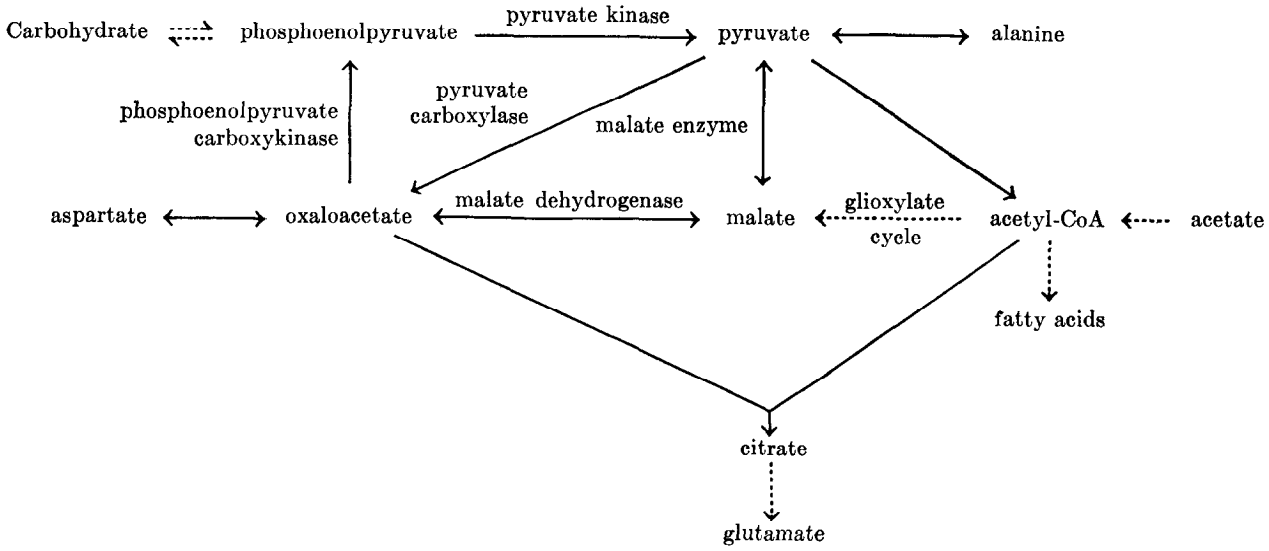
Cannata and Stoppani (1) concluded that phosphoenolpyruvate carboxykinase seemed to be the enzyme responsible for carbon dioxide fixation in bakers' yeast, since their enzyme was inactive in the presence of pyruvate and ATP and their attempts to demonstrate a malate enzyme in cell-free preparations were unsuccessful. These conclusions are not supported by the results reported here which indicate that phosphoenolpyruvate carboxykinase is involved in the decarboxylation of oxaloacetate to phosphoenolpyruvate whereas pyruvate carboxylase is responsible for the carboxylation of pyruvate to oxaloacetate. According to the data presented in Table V, the pathways in which pyruvate carboxylase, pyruvate kinase, phosphoenolpyruvate carboxykinase, and malate enzyme are involved in yeast metabolism have been schematically represented in Diagram 1. When glucogenesis was active (cells grown on pyruvate, malate, aspartate, or acetate), the level of pyruvate kinase decreased drastically whereas that of phosphoenolpyruvate carboxykinase increased greatly. This indicates that the formation of phosphoenolpyruvate from any of the compounds utilized did not occur from pyruvate through the reversal of the glycolytic reaction catalyzed by pyruvate kinase, but from oxaloacetate through the action of phosphoenolpyruvate carboxykinase. These results are in agreement with the thesis advanced by Utter, Keech, and Scrutton (16) that pyruvate kinase probably does not play a major role in the synthesis of phosphoenolpyruvate from pyruvate during gluconeogenesis in chicken liver and with the observations of Shrago *et al.* (23) and Lardy (24) that phosphoenolpyruvate carboxykinase activity in rat liver is closely correlated with carbohydrate formation.

By contrast, when glycolysis was operative (cells grown on glucose), the level of pyruvate kinase became very high and that of phosphoenolpyruvate carboxykinase decreased remarkably. This seems to indicate that oxaloacetate was not synthesized from phosphoenolpyruvate by phosphoenolpyruvate carboxykinase but rather from pyruvate by pyruvate carboxylase since under these growth conditions no malate enzyme was detectable in the cells. Therefore, pyruvate seems to be the immediate precursor of oxaloacetate for both the operation of the Krebs cycle and the biosynthesis of carbon compounds. In fact, the level of pyruvate carboxylase reached its maximal value when glucose or pyruvate was used as the carbon sources. An increase in pyruvate carboxylase activity in rat liver has been observed before by Wagle (25) in diabetic animals and by Henning, Seiffert, and Seubert (26) after cortisol administration. Our data showing that pyruvate kinase is essential in glycolysis but not in gluconeogenesis are in complete harmony with those just published by Krebs and Eggleston (27). These authors found that, on changing from a standard diet to a low carbohydrate diet, the activity of pyruvate kinase in rat liver fell to about one-third, and on changing to a high carbohydrate diet, it rose more than 3-fold.

With respect to the function of the inducible malate enzyme it is evident from the reported data that, when the cells were grown on malic acid, this enzyme was synthesized in great

DIAGRAM 1

Metabolic pathways of pyruvate in yeast



amounts, very likely in order directly to provide pyruvate, the inevitable precursor of acetyl-CoA and alanine. Indirect formation of pyruvate without the involvement of the malate enzyme is also possible through the combined action of malate dehydrogenase, phosphoenolpyruvate carboxykinase, and pyruvate kinase. Analogously, with aspartate as substrate, pyruvate can be formed from oxaloacetate through malate by malate dehydrogenase and malate enzyme or through phosphoenolpyruvate by phosphoenolpyruvate carboxykinase and pyruvate kinase. The fact that, in repeated experiments with aspartate as substrate, malate enzyme increased proportionally when phosphoenolpyruvate carboxykinase and pyruvate kinase decreased and vice versa seems to suggest that both pathways may be operative. Finally, it cannot yet be excluded that oxaloacetate may be converted directly to pyruvate since malate enzyme has been shown to catalyze also the decarboxylation of the former ketoacid to pyruvic acid (28).

The induction of malate enzyme in *R. glutinis* when the yeast was grown on acetate may be explained by the accumulation of malate resulting from the operation of the glyoxylate cycle (29). Since *H. anomala* grew perfectly well on acetate and did not contain under these conditions detectable amounts of malate enzyme, it seems very likely that this enzyme is not essential for gluconeogenesis in this yeast. Shrago *et al.* (23) and Lardy *et al.* (24) had previously concluded that the changes in malate enzyme in rat liver do not support the contention that this enzyme is directly involved in carbohydrate synthesis from pyruvate.

The discovery of the essential function of the biotin-containing enzyme, pyruvate carboxylase, in the formation of oxaloacetate (and consequently of aspartate) from pyruvate in yeast may explain why aspartic acid can to some extent compensate for biotin deficiency in yeast (30–32).

SUMMARY

Pyruvate carboxylase, the enzyme which catalyzes the formation of oxaloacetate from pyruvate, adenosine triphosphate, and CO_2, in the presence of Mg^{++}, has been purified from bakers' yeast. The reaction is stimulated by the presence of acetyl coenzyme A or coenzyme A and by K^+ ions. The enzyme is completely inhibited by avidin. Biotin prevents this inhibition. Oxalate has been found to be a strong noncompetitive inhibitor of the reaction. The lability of the enzyme, the affinities of the various substrates, activators and inhibitors for the enzyme, and the effect of pH have also been investigated.

The activities of pyruvate carboxylase and related enzymes have been determined in two yeast species grown in media containing different carbon sources. Pyruvate kinase was high only when glycolysis was active, whereas phosphoenolpyruvate carboxykinase increased when gluconeogenesis was operative. Pyruvate carboxylase varied only slightly with the carbon compound consumed by the cells. The malate enzyme appeared extremely active after culturing the cells in malic acid or other malate precursors. The essential function of pyruvate carboxylase in yeast grown on glucose seems to be to provide oxaloacetate for both the operation of the Krebs cycle and the biosynthesis of carbon compounds.

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