Some Aspects of the Kinetics of Rat Liver Pyruvate Carboxylase

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1. The kinetics of rat liver pyruvate carboxylase were examined and the effect of various agents as activators or inhibitors determined. 2. Essentially similar results were obtained in comparisons of kinetics determined by a radioactivity method involving extracts of acetone-dried powders from whole livers and with a spectrophotometric assay using partially purified enzyme from the mitochondrial fraction. Activity per g of liver from fed or starved rats assayed under optimum substrate and activator conditions was 3 or 6 μmol of oxaloacetate formed/min at 30°C, respectively. 3. The enzyme exhibited cold-lability and lost activity on standing, even in 1.5M-sucrose. 4. The K_m towards pyruvate was about 0.33mM and towards bicarbonate 4.2mM. K_m towards MgATP^{2-} was 0.14mM. Mg^{2+} ions activated the enzyme, in addition to their role in MgATP^{2-} formation. From calculations of likely concentrations of free Mg^{2+} in the assay medium a K_a towards Mg^{2+} of about 0.25mM was deduced. Mn^{2+} also activated the enzyme as well as Mg^{2+} , but at much lower concentrations. It appeared to be inhibitory when concentrations of free Mn^{2+} as low as 0.1mM were present. 5. Excess of ATP is inhibitory, and this appears at least in part independent of the trapping of Mg^{2+} . 6. Both Co^{2+} and Zn^{2+} were inhibitory; 2mM of the latter appeared to be bound even in the presence of excess of Mg^{2+} and the inhibition was time-dependent. 7. Ca^{2+} inhibited by competition with Mg^{2+} (K_i about 0.38mM). The inhibition due to Ca^{2+} was less pronounced when activation was with Mn^{2+} . Inhibition by Ca^{2+} and ATP appeared to be additive. 8. Hill plots suggested that no interactions occurred between ATP-binding sites. Although similar plots for total Mg^{2+} gave $n = 3.6$, no conclusions could be drawn due to the chelation of the cation with other components of the assay. Similar difficulties arose in assessing the values for Ca^{2+} . 9. The enzyme was inactive in the absence of acetyl-CoA and showed a sigmoidal response in its presence. K_a was about 0.1mM with possibly up to four binding sites. Malonyl-CoA was a competitive inhibitor, with K_i 0.01mM. 10. There was no apparent inhibition by glucose, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, acetoacetate, β -hydroxybutyrate, malate, aspartate, pyruvate, palmitoylcarnitine, octanoate, glutathione, butacaine, triethyltin or potassium chloride under the conditions used. Inhibition was found with citrate (possibly by chelation) and adenosine, and also by phosphoenolpyruvate, AMP, ADP and cyclic AMP, K_i towards the last four being 0.55, 0.76, 0.25 and 1.4mM respectively.

Pyruvate carboxylase [pyruvate-carbon dioxide ligase (ADP), EC 6.4.1.1] is the first enzyme on the gluconeogenic pathway from C_3 precursors, carboxylating pyruvate to oxaloacetate. Although many reports have been published on the kinetic behaviour of this enzyme from yeast (Cazzulo & Stoppani, 1969a,b), chicken liver (Utter & Keech, 1963; Scrutton & Utter, 1967) and in sheep kidney (Ling & Keech, 1966; Keech & Barritt, 1967), relatively few results have been presented on the behaviour of the enzyme from rat liver, despite numerous studies on the control of gluconeogenesis in this tissue. Alterations in the rate of glucose production are often discussed in the

context of the information available for the avian liver enzyme. However, Seubert, Henning, Schoner & L'Age (1968) have shown that the acetyl-CoA requirement of the rat enzyme is much higher than that of the chicken and nearer the range of the hepatic concentration of this substrate. It was therefore decided that a more detailed study of rat liver pyruvate carboxylase was justified, particularly with a view to making more appropriate comparisons with the rates of gluconeogenesis.

Pyruvate carboxylase has been assayed by two procedures, first by incorporation of $^{14}\text{CO}_2$ into oxaloacetate, and secondly by following spectrophotometrically the removal of oxaloacetate

formed with malate dehydrogenase and NADH (Utter & Keech, 1963). The second procedure is possible only when the pyruvate carboxylase is purified from contaminating lactate dehydrogenase. Since the initial purpose of the present work was to enable measurements of pyruvate carboxylase to be made on samples of liver derived from perfusion experiments etc., in which insufficient material was available to carry out purification, the radioactivity assay was mandatory. However, for comparative purposes the enzyme has also been extracted from mitochondrial preparations and the spectrophotometric assay was then used. The bulk of the pyruvate carboxylase activity in rat liver is believed to be mitochondrial, but there is evidence that some extramitochondrial activity may exist (Seubert *et al.* 1968).

MATERIALS AND METHODS

Animals. Male albino Wistar rats bred in this department were used.

Materials. ATP (disodium salt), sodium pyruvate, CoA, NADH, potassium lithium acetyl phosphate, phosphotransacetylase, citrate synthase, malate dehydrogenase, AMP, 3':5'-cyclic AMP, ADP (trisodium salt), adenosine, phosphoenolpyruvate (potassium salt), glucose 6-phosphate and fructose 1,6-diphosphate were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Tris (Trizma base) was purchased from Sigma (London) Chemical Co., London S.W.6, U.K. and bovine plasma albumin was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Sodium [^{14}C]bicarbonate (44 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Radioactivity assay

Preparation of extract. Liver was homogenized in 9 vol. of acetone cooled on solid CO_2 and the precipitate was collected by filtration at room temperature. The powder obtained was dried for 2 h *in vacuo* over P_2O_5 . Shortly before assay, the powder was homogenized at 30°C in 9 vol. of 125 mM-tris-HCl buffer, pH 7.4. The extract was diluted further 1:1.5 or 1:2. The tris buffer was boiled and gassed with N_2 before use to minimize the content of dissolved CO_2 .

Incubation medium. The incubation medium used was based on those of Henning & Seubert (1964) and Felicioli, Gabrielli & Rossi (1967). Samples (0.1 ml) of extract were incubated for 0, 2, 4, and 6 min with 0.2 ml of the assay medium [final concns. 125 mM-tris-HCl, 7.5 mM-sodium pyruvate, 8.3 mM- MgSO_4 , 2.2 mM-ATP, 0.83 mM-CoA, 0.83 mM-acetyl phosphate, 10 units of phosphotransacetylase, 0.5 unit of citrate synthase and 100 mM- $\text{NaH}^{14}\text{CO}_3$ (approx. 20000 c.p.m./ μmol), except insofar as the concentration of one of the additions was specifically varied; the final pH was 7.2 at 30°C]. The reaction was stopped by the addition of 0.5 ml of ice-cold 10% (w/v) trichloroacetic acid. The mixture was gassed with CO_2 for 10 min to remove $^{14}\text{CO}_2$ and the protein precipitate was removed by centrifugation. Portions of the super-

natant were added to scintillation fluid [0.4% 2,5-bis-(5-tert.-butylbenzoxazol-2-yl)thiophen and 8% naphthalene in methylCellosolve-toluene (2:3, v/v)] and the radioactivity was counted at 60–70% efficiency.

The initial velocity of the reaction was estimated from the amount of incorporation at the time-intervals stated. Activity observed in the absence of pyruvate in the medium (possibly due to acetyl-CoA carboxylase; never more than 5% of that in the presence of pyruvate) was subtracted.

Spectrophotometric assay

Preparation of extract. Mitochondria (given by Dr E. J. Harris) were prepared from fresh liver by homogenization in 9 vol. of 0.25 M-sucrose containing 0.05% bovine serum albumin and 1 mM-ethylenedioxybis(ethylamine)tetraacetate. After the initial centrifugation to remove nuclei and cell debris, the supernatant was centrifuged for 5 min at 10000g. The pellet was resuspended and washed three times in 0.16 M-sucrose containing 42 mM-KCl, 1 mM-ethylenedioxybis(ethylamine)tetraacetate and 0.05% albumin. After a final wash in water the pellet was freeze-dried from not more than 10 ml of water.

Extraction of pyruvate carboxylase. This was based on the method of Keech & Utter (1963). Freeze-dried mitochondria were extracted at room temperature with 5–6 ml of 125 mM-tris-HCl buffer, pH 7.4. Insoluble matter was removed by centrifugation at 20000g for 10 min. The supernatant was made 16% saturated with $(\text{NH}_4)_2\text{SO}_4$, precipitated material being removed by centrifugation at 20000g for 20 min. The concentration of $(\text{NH}_4)_2\text{SO}_4$ was then increased to 38% saturation. The precipitate was collected by centrifugation at 20000g for 10 min and resuspended at 30°C in the minimum volume of 50% (w/v) sucrose, in which it is reputedly stable (Scrutton & Utter, 1965); the protein concentration (less than 1 mg/ml) was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Incubation medium. The assay medium used was that described by Utter & Keech (1963) and contained (final concns.): 112 mM-tris-HCl, 2 mM-ATP, 8 mM- MgSO_4 , 20 mM- NaHCO_3 , 2 mM-sodium pyruvate, 0.2 mM-NADH, 0.24 mM-acetyl-CoA (prepared *in situ* from CoA, acetyl phosphate and phosphotransacetylase) and 2.2 units of malate dehydrogenase; the final pH was 7.2 at 30°C . The reaction was initiated by the addition of 0.1 ml of enzyme extract to 0.9 ml of assay medium. The rate of utilization of NADH was followed for about 10 min by the decline in E_{340} in a Unicam SP.800 spectrophotometer. As a control, the rate of loss of NADH in the absence of acetyl-CoA was followed and compared with the activity in its presence. The former was a variable proportion of the total activity, depending on the mitochondrial preparation. Since it was mainly pyruvate-dependent it probably represented contamination by lactate dehydrogenase. The concentration of acetyl-CoA was assayed by the method of Michal & Bergmeyer (1962) and the value of 0.24 mM included in the constituents results from this.

RESULTS

Pyruvate- and bicarbonate-dependence. Work presented below was carried out under two basically different conditions: either by use of a homogenate,

Fig. 1. Reciprocal plots of the activity of rat liver pyruvate carboxylase at different concentrations of NaHCO_3 (a) and (c) and pyruvate (b) and (d). (a) and (b), $(\text{NH}_4)_2\text{SO}_4$ -fractionated extracts of freeze-dried mitochondria; (c) and (d) extracts of acetone-dried powders of whole liver. The mitochondrial enzyme was assayed spectrophotometrically: units are μmol of NADH oxidized/min per mg of protein; acetone-dried powder extracts were assayed by following the incorporation of $\text{NaH}^{14}\text{CO}_3$ into citrate: units are μmol of citrate formed/min per g of liver.

in which case enzyme activity was measured by incorporation of ^{14}C into oxaloacetate, or with mitochondrial extracts, activity being measured spectrophotometrically. Media described in the Materials and Methods section found to be optimum for activity contained magnesium sulphate at 7–10 mM and ATP at 1–2 mM. Under these conditions the activity of the homogenate was linear with time for at least 6 min and was equivalent to a capacity of 3–6 μmol of product formed/min per g of liver at 30°C. In the presence of these concentrations of Mg^{2+} and ATP, the K_m towards pyruvate was approx. 0.33 mM, and towards bicarbonate, 4.2 mM (Fig. 1). Essentially no difference was found in these (or any other) parameters between the homogenate and the mitochondrial extract assayed by the different procedures. Scrutton & Utter (1965) also found no change in kinetic parameters on purification. Keech & Utter (1963) found the K_m for the chicken liver enzyme to be 0.44 mM towards pyruvate and 0.98 mM towards bicarbonate at pH 7.8, when the proportion of the bicarbonate as CO_3^{2-} is larger than under our conditions. At pH 7 the $[\text{HCO}_3^-]/[\text{CO}_3^{2-}]$ ratio is large so that, if HCO_3^- is the true substrate, the K_m value we find is perhaps nearer 2 mM. McLure (1969) found

for the purified rat liver enzyme a K_m for pyruvate of 0.1 mM and for bicarbonate of 1.5 mM. Others have commented on the difficulty of demonstrating bicarbonate-dependence because of the technical problem of removing all extraneous CO_2 ; it is satisfactory therefore that the two different assays gave similar results. Scrutton, Keech & Utter (1965) and Taylor, Nielson & Keech (1969) found that at concentrations of pyruvate greater than 1 mM the K_m towards the substrate increased appreciably. Our results (Fig. 1) give no hint of this at concentrations up to 3 mM.

Pyruvate carboxylases from different sources have reported pH optima of 8.4 for the sheep kidney enzyme (Ling & Keech, 1966), 7.6 for the chicken liver enzyme (Keech & Utter, 1963) and 8.3 for the yeast enzyme (Ruiz-Amil, de Torrontegui, Palacián, Catalina & Losada, 1965). Using the radioactivity assay, we found the rat liver enzyme had a broad pH optimum at 7.2–7.4 (at 30°C); subsequent studies were made at pH 7.2.

Cold-lability of the enzyme. The majority of the pyruvate carboxylases from several of the sources so far studied are cold-labile, and we have found this to be so for the rat liver enzyme. The enzyme extracted from mitochondria and kept at room

temperature (20–23°C) in 1.5M-sucrose loses approx. 50% of its activity overnight, and 75% after three days. If the liver is stored intact at –80°C loss of activity is somewhat slower, declining to 50% only after 10–15 days. Storage of acetone-dried powders of liver either at 0°C or room temperature resulted in loss of activity overnight, and once the enzyme had been extracted into tris-HCl buffer, pH 7.4 (as described in the Materials and Methods section), the activity of the preparation decreased within 3 h even if maintained at 30°C. It is possible that further purification would have increased stability

in 1.5M-sucrose (Scrutton & Utter, 1965; Ling & Keech, 1966; McLure, 1969).

Effect of Mg^{2+} and Mn^{2+} . At a 2mM concentration of ATP, variation of activity with changing $[Mg^{2+}]$ gives a sigmoidal plot (Fig. 2). Slight inhibition of activity was observed at the highest concentrations studied; a similar phenomenon was noted with the chicken liver enzyme by Keech & Utter (1963). Mn^{2+} gave a slightly higher activity than that attained with Mg^{2+} and at a considerably lower concentration, in contrast with the findings of Keech & Utter (1963). Further addition of Mn^{2+} brought about progressive inhibition. The phenomenon of optimum activation by Mn^{2+} at lower concentrations than those at which Mg^{2+} activates, followed by an equally pronounced inhibition, is fairly general and exactly similar patterns are seen with, e.g., phosphoenolpyruvate carboxykinase (Foster, Lardy, Ray & Johnson, 1967) and RNA polymerase (Roeder & Rutter, 1969).

A Hill plot of the data in Fig. 2 gives a value of 3.6 for the apparent number of binding sites for Mg^{2+} , but this is almost certainly misleading since it is based on the total $[Mg^{2+}]$ in the presence of ATP. However, the Hill plot can be used to provide an estimate (3.0mM) of the apparent K_a towards total Mg^{2+} that compares with results of 2.8mM quoted for the chicken liver enzyme (Scrutton & Utter, 1965), 4.2mM for the yeast enzyme (Ruiz-Amil *et al.* 1965), 2.5mM for the sheep kidney enzyme (Ling & Keech, 1966) and 3.0mM for the rat liver enzyme (McLure, 1969).

In an attempt to get a more meaningful value of K_a towards Mg^{2+} , we have made calculations of the extent of complex-formation with anions under the conditions employed (Table 1). This is of necessity approximate because of uncertainties in the values of the stability constants used. It has required the

Fig. 2. Effect of various concentrations of $MnCl_2$ ($\blacktriangle$) and $MgSO_4$ ($\bullet$) on the activity of mitochondrial pyruvate carboxylase, assayed spectrophotometrically.

Table 1. Concentrations of Mg^{2+} , ATP^{4-} and $MgATP^{2-}$ at different total concentrations of ATP and magnesium sulphate

Calculations were carried out with a computer program appropriate for solving equations arising from multiple equilibria. Complex-formation between Mg^{2+} and SO_3^{2-} , CO_3^{2-} , and Cl^- was also taken into account. Stability constants assumed for Mg complexes were (M^{-1}): $MgATP^{2-}$, 20000; $MgSO_4$, 160; $MgCO_3$, 150; $MgCl^+$, 3.5.

[ATP] _t (mM)	[MgSO ₄] _t 8 mM			[MgSO ₄] _t 4 mM		
	ATP ⁴⁻ (mM)	Mg ²⁺ (mM)	MgATP ²⁻ (mM)	ATP ⁴⁻ (mM)	Mg ²⁺ (mM)	MgATP ²⁻ (mM)
0.5	0.01	1.10	0.49	0.01	0.52	0.49
1.0	0.01	1.01	0.99	0.02	0.44	0.98
1.5	0.02	0.92	1.48	0.05	0.37	1.45
2.0	0.02	0.82	1.98	0.08	0.30	1.92
2.5	0.04	0.78	2.46	0.13	0.23	2.37
3.0	0.05	0.71	2.95	0.20	0.17	2.80
4.0	0.09	0.56	3.91	0.55	0.08	3.45
5.0	0.14	0.43	4.86	1.26	0.04	3.74
6.0	0.24	0.30	5.76	2.16	0.02	3.84

Fig. 3. Variation of activity (●) of pyruvate carboxylase with different concentrations of ATP and Mg^{2+} . (a), (b) and (c) Effect of changing $[Mg^{2+}]$, keeping $[ATP]$ at 2, 1 and 0.2 mM respectively; (d), (e) and (f) effect of changing $[ATP]$ with $[Mg^{2+}]$ at 8, 4 and 2 mM respectively. ·····, $[MgATP^{2-}]$; ----, $[ATP^{4-}]$; - · - · - ·, $[Mg^{2+}]$; —, enzyme activity.

taking into account of formation of magnesium sulphate, magnesium carbonate and $MgCl^+$ (Gryzbowski, Tate & Datta, 1970) as well as of $MgATP^{2-}$. Although it is now possible to plot activity versus $[Mg^{2+}]$ (Fig. 3a), the most important points for the estimation of K_a are for conditions in which $MgATP^{2-}$ is also varying. Nevertheless it is possible to say that the K_a towards Mg^{2+} is likely to be not greater than 0.25 mM.

When $[ATP]$ was kept constant at 1 or 0.2 mM, variation of activity with different Mg^{2+} concentrations was less sigmoidal than before (Figs. 3b and 3c). Since with the lower concentrations of ATP the amount of $MgATP^{2-}$ is fairly constant over the range studied, it is possible to assess the K_a towards Mg^{2+} from the quantity of this ion calculated to be present at half-maximal velocity; this gives a value of 0.25 mM, in fair agreement with that obtained from Fig. 3(a). Clearly Mg^{2+} has a twofold effect: that of forming $MgATP^{2-}$, and as Mg^{2+} itself. Until $[Mg^{2+}]$ exceeds $[ATP]$ there is little free Mg^{2+} , available hence the sigmoidal shape of Fig. 2(a) for variation of activity with changing $[Mg^{2+}]$.

If the effects of Mg^{2+} can be so explained, those of Mn^{2+} must be different. The stability constant for $MnATP^{2-}$ formation is slightly greater than for $MgATP^{2-}$. Presumably in the range 0–2 mM Mn^{2+} is activating, in part through increases in substrate (i.e. $MnATP^{2-}$) formation, and any activation resulting from binding of Mn^{2+} to the enzyme must

have a K_a several orders of magnitude lower than for Mg^{2+} since there is so little free Mn^{2+} present. As soon as $[free\ Mn^{2+}]$ rises appreciably, i.e. when the total is above 2 mM, inhibition rapidly sets in. Thus appreciable quantities of free Mn^{2+} appear to be inhibitory, though this inhibition can apparently be offset when adequate Mg^{2+} is present (see below).

Effect of ATP. In the presence of excess of Mg^{2+} (8 mM) the maximum activity was reached with an ATP concentration between 1 and 2 mM (Fig. 4). At higher concentrations ATP was inhibitory; this could result from the chelation of Mg^{2+} . If the K_a towards Mg^{2+} is 0.25 mM, this is unlikely to be of consequence until $[Mg^{2+}]$ falls to less than, say, 1 mM, but this occurs when $[ATP]$ is greater than 1 mM (Table 1). On the other hand, pyruvate carboxylase may be subject to inhibition by ATP^{4-} (Blair, 1969) whose concentration when the total amount of ATP added (ATP_t) is 6 mM is 0.24 mM (Table 2). The effects of ATP at various Mg^{2+} concentrations are also shown in Figs. 3(d)–3(f).

A Hill plot derived from the combined results for the spectrophotometric assays (Fig. 3d–3f) gives n , the apparent number of interacting binding sites, as 1.0 ($r = 0.98$), i.e. no interactions. This is in contrast with the results of Keech & Barritt (1967) with sheep kidney enzyme, but this difference is due to the different Mg^{2+} concentrations used. Both these authors, and Cazzulo & Stoppani (1969a,b) working with yeast, have found that the addition

of increasing amounts of Mg^{2+} lowers the apparent binding constant for ATP from 1.7 and 1.3 for the two sources respectively, when assayed at equimolar concentrations of Mg^{2+} and ATP, to 1.0 when excess of Mg^{2+} is present. Apparent interaction with equimolar concentrations of ATP and Mg^{2+} presumably arises because the binding of Mg^{2+} elsewhere means that $[MgATP^{2-}]$ is appreciably less than $[ATP]$.

As shown in Tables 1 and 2, when the concentration of magnesium sulphate is greater than that of ATP, the latter is mainly as $MgATP^{2-}$. The apparent K_m for ATP obtained is therefore an estimate of the K_m for the complex ion, and the value of 0.14 mm at pH 7.2 is in good agreement with that of McLure (1969) of 0.1 mm at pH 7.4, though somewhat higher than the value of 0.03 mm found by Keech & Utter (1963).

When $[Mg^{2+}]$ was held constant at lower values the inhibition as $[ATP]$ increased became more marked (Figs. 3*d*–3*f*). This is consistent with inhibition through removal of Mg^{2+} but could of

Fig. 4. Activity of mitochondrial pyruvate carboxylase assayed spectrophotometrically, with various concentrations of ATP (8 mm- $MgSO_4$).

course also result alternatively or additionally from inhibition by ATP^{4-} . The results in Table 2 are interpreted to suggest that there is inhibition from both sources. Thus with total Mg^{2+} concentrations $[Mg^{2+}]_t$ of 4 and 8 mm and $[ATP]_t$ of 2 and 6 mm, $[Mg^{2+}]$ is about 0.3 mm but $[ATP^{4-}]$ is about four times as much in the second case and the inhibition 30% as opposed to 16%. On the other hand, when $[Mg]_t$ is 4 and 2 mm and $[ATP]_t$ 2 and 1.8 mm, $[ATP^{4-}]$ is the same but $[Mg^{2+}]$ is 0.3 and 0.08 mm, and the activity is 84 and 58% of that with respect to $[Mg]_t$ of 8 mm and $[ATP]_t$ 2 mm ($[Mg^{2+}]$ 0.87 mm, $[MgATP^{2-}]$ 1.89 mm).

Inhibition by bivalent cations. Ion antagonism is a common phenomenon with metal-activated enzymes, and is particularly prevalent between metal ions in the same group, e.g. Mg^{2+} and Ca^{2+} . Kimmich & Rasmussen (1969) have reported that rat liver mitochondrial pyruvate carboxylase is inhibited by Ca^{2+} , the extent of inhibition depending on the $[Mg^{2+}]/[Ca^{2+}]$ ratio.

Addition of calcium chloride to media containing 2 mm-ATP and 3.2 or 8 mm-magnesium sulphate produced marked inhibition of the mitochondrial pyruvate carboxylase (Fig. 5*a*). The Dixon plot at the higher concentrations of magnesium sulphate was linear, but at lower concentrations it became concave upwards as the concentration of Ca^{2+} increased (Fig. 5*b*). Extrapolation of the linear portion of the plot indicates competition between Mg^{2+} and Ca^{2+} , with K_i for calcium chloride 0.38 mm. Hill plots of the results show a value of n for Ca^{2+} rising from 1 in conditions of saturating $[Mg^{2+}]$ to 1.4 when the concentration of magnesium sulphate is decreased to 3.2 mm. Butacaine (Chance, Azzi, Mela, Radda & Vainio, 1969) at 1 mm is unable to relieve the inhibition produced by Ca^{2+} . At the lower $[Mg^{2+}]$ Ca^{2+} will displace Mg^{2+} from binding to ATP and the modes of Ca^{2+} inhibition will then be multiple. It is not surprising therefore that the inhibitions produced by Ca^{2+} and by ATP are found to be additive (Table 3). The Dixon plot of Ca^{2+} inhibition at high concentrations of ATP, like that at low concentrations of magnesium sulphate, is non-linear, the straight portion at low concentra-

Table 2. Activity of pyruvate carboxylase in the presence of various concentrations of magnesium sulphate and ATP

Inhibition is expressed with respect to activity at 2 mm-ATP and 8 mm- $MgSO_4$.

Concns. added		Calc. concn. present			Inhibition (%)
$MgSO_4$ (mm)	ATP (mm)	Mg^{2+} (mm)	$MgATP^{2-}$ (mm)	ATP^{4-} (mm)	
4	2	0.3	1.92	0.05	16
8	6	0.3	5.80	0.20	30
2	1.8	0.08	1.53	0.05	42

Fig. 5. (a) Inhibition of mitochondrial pyruvate carboxylase by CaCl_2 ; $\bullet$ 2mM-ATP, 8mM- MgSO_4 ; $\blacktriangle$, 2mM-ATP, 3.2mM- MgSO_4 ; $\blacksquare$, 4mM-ATP, 8mM- MgSO_4 . (b) Dixon plot of the results in (a).

Table 3. *Effects of Mg^{2+} , Mn^{2+} , Ca^{2+} and ATP on the activity of pyruvate carboxylase*

The inhibition by ATP is expressed relative to the specific activity of the enzyme at 8mM- MgSO_4 and 2mM-ATP. Higher concentrations of ATP are inhibitory (Fig. 2).

	Additions (mM)		Inhibition (%)			Theoretical if additive
	ATP	Ca^{2+}	by Ca^{2+}	by ATP	by Ca^{2+} + ATP	
(a) MgSO_4 (8mM) ...	2	—				
	2	0.45	42			
	4	—		36		
	4	0.45			59	57
(b) MnCl_2 (2mM) ...	2	—				
	2	0.45	15			
	4	—		56		
	4	0.45			68	63

tions of Ca^{2+} intersecting at the same point as the plots with 2mM-ATP (Fig. 5b).

Pyruvate carboxylase is less susceptible to inhibition by Ca^{2+} when activated by Mn^{2+} , 1mM-calcium chloride producing only 20% inhibition (Fig. 6) as opposed to 60% in the Mg^{2+} -activated system. Under these conditions the enzyme is more susceptible to inhibition by ATP, an additional 2mM producing 55% inhibition as opposed to 25%. The effect of Ca^{2+} and ATP was again additive (Table 3). From the Hill plot of the data, n for Ca^{2+} was less than 1 for the Mn^{2+} -activated system.

Unlike the chicken liver enzyme, which is activated by Co^{2+} (Keech & Utter, 1963), rat liver pyruvate carboxylase is inhibited by Co^{2+} (Fig. 7). In this respect it resembles the enzyme from yeast

(Cazzulo & Stoppani, 1967). Inhibition was produced in the presence of 8mM-magnesium sulphate by concentrations of Co^{2+} similar to those of Ca^{2+} found to be inhibitory. However with both Ca^{2+} in the presence of Mn^{2+} and Co^{2+} in the presence of Mg^{2+} inhibition appeared to be only partial (Figs. 6 and 7).

Zn^{2+} was a potent inhibitor of rat liver pyruvate carboxylase, complete loss of activity being produced by 0.06mM-zinc sulphate in the presence of 2mM-ATP and 8mM-magnesium sulphate (Fig. 7). A Hill plot of the results suggests the involvement of two Zn^{2+} ions even at saturating concentrations of Mg^{2+} . Prior addition of GSH to the enzyme prevents loss of activity, probably by chelation of the Zn^{2+} . Addition of GSH at 2.6mM to the normal assay mixture has no effect on the activity of rat liver

Fig. 6. Inhibition of pyruvate carboxylase by Ca^{2+} , with Mn^{2+} as the activating ion. ●, 2mM-ATP, 2mM- MnCl_2 ; ▲, 4mM-ATP, 2mM- MnCl_2 .

Fig. 7. Inhibition of mitochondrial pyruvate carboxylase by CoCl_2 (□) or ZnSO_4 (○) (2mM-ATP, 8mM- MgSO_4).

pyruvate carboxylase, unlike the enzyme derived from yeast, which is activated (Gailiusis, Rinne & Benedict, 1964). The inhibition of pyruvate carboxylase by Zn^{2+} increases with increasing time of preincubation, is not reversed by dilution and probably involves irreversible binding of the metal ion to the enzyme protein.

ADP is an inhibitor of pyruvate carboxylase (Keech & Utter, 1963), and as Table 4 shows, the inhibition of the rat liver enzyme is only partially relieved by the addition of further magnesium sulphate. As with ATP, inhibitory effects of Ca^{2+} and ADP were additive (Table 4). Except for the assay at 2mM-magnesium sulphate, 1.8mM-ADP and 0.2mM-ATP, the percentage inhibition produced by Ca^{2+} was the same in the presence or absence of ADP.

Acetyl-CoA dependence. The enzyme from rat liver is virtually inactive in the absence of acetyl-CoA and shows a sigmoidal response to addition of the activator (Fig. 8a), a situation encountered in other studies with rat liver, chicken liver and sheep kidney enzyme (Seubert *et al.* 1968; Scrutton & Utter, 1965; Ling & Keech, 1966). Neither CoA nor cyclic AMP, both activators of the yeast enzyme where the requirement for acetyl-CoA is not absolute (Cooper & Benedict, 1968; Cazzulo & Stoppani, 1968), are effective. From the Hill plot of the results in Fig. 8(a) (Fig. 8c) n , the apparent number of binding sites, is 3.3; this is suggestive of four actual sites but with co-operative interactions facilitating the binding of successive molecules of acetyl-CoA in a manner analogous to the postulated binding of AMP to fructose 1,6-diphosphatase (Taketa & Pogell, 1965). At concentrations of activator less than 0.04mM, the slope of the Hill plot falls towards 1, the opposite of the situation reported for the chicken liver enzyme.

The range of greatest change in activity with changing concentrations of acetyl-CoA is 0.03–0.15mM, in good agreement with the results of Henning & Seubert (1964), but higher than the K_a value found by McLure (1969) of 0.04mM. However, this latter report also states that the parameter is pH-sensitive over the range pH 7–8, the K_a cited being assayed at pH 7.4. K_a for the rat liver enzyme was estimated from a plot of $1/v$ versus $1/[\text{acetyl-CoA}]^3$ (Fig. 8d) as suggested by Lineweaver & Burk (1934) for reactions involving multiple binding of the activator or substrate; at no point was the more usual $1/v$ versus $1/[\text{acetyl-CoA}]$ plot linear (Fig. 8b). The value found for K_a of 0.1mM is appreciably higher than that for the chicken liver enzyme (Scrutton & Utter, 1965) but it is closer to the recorded physiological values for acetyl-CoA in rat liver (Start & Newsholme, 1968).

Inhibition by malonyl-CoA. As with the chicken liver enzyme (Scrutton & Utter, 1967), rat liver pyruvate carboxylase is inhibited by malonyl-CoA, with K_i 0.01mM (Fig. 9). The inhibition is competitive with respect to acetyl-CoA, high concentrations of the latter giving almost complete protection. From a Hill plot, only 1 molecule appears to be bound at 0.05mM, falling to 0.5 at 0.24mM-acetyl-CoA.

Inhibitors of pyruvate carboxylase. Of the compounds tested as activators or inhibitors of rat liver pyruvate carboxylase, octanoate at 4.2mM, an inhibitor of certain glycolytic enzymes (Weber, Lea, Convery & Stamm, 1967), had no effect, and of the glycolytic intermediates tested, only phosphoenolpyruvate was an effective inhibitor. β -Hydroxybutyrate, up to 30mM, and acetoacetate (8mM), both of which are increased in ketosis (Wieland, 1968), also had no effect. Inhibition by

Table 4. *Effects of Mg²⁺, ATP and ADP on the activity of pyruvate carboxylase*

Basal activity was different in different experiments.

	Additions (mM)			Specific activity ($\mu\text{mol/min per}$ mg of protein, 30°C)	Inhibition (%)			
	ATP	ADP	Ca ²⁺		by Ca ²⁺	by ADP	by Ca ²⁺ + ADP	theoretical if additive
MgSO ₄ (8 mM) ...	2.0	—	—	0.59				
	2.0	—	0.45	0.38	35			
	1.0	—	—	0.56				
	1.0	—	0.45	0.43	24			
	1.0	1.0	—	0.47		16		
	1.0	1.0	0.45	0.33	30		41	36
	0.5	—	—	0.53				
	0.5	—	0.45	0.40	34			
	0.5	1.5	—	0.36		33		
	0.5	1.5	0.45	0.26	33		53	49
	0.2	—	—	0.39				
	0.2	—	0.45	0.31	22			
	0.2	1.8	—	0.17		56		
	0.2	1.8	0.45	0.13	25		67	66
MgSO ₄ (8 mM) ...	2.0	—	—	0.139				
	2.0	—	0.45	0.097	33			
MgSO ₄ (4 mM) ...	2.0	—	—	0.098				
	2.0	—	0.45	0.045	53			
	1.0	—	—	0.118				
	1.0	—	0.45	0.071	40			
	1.0	1.0	—	0.080		32		
	1.0	1.0	0.45	0.051	36		57	59
	0.7	—	—	0.118				
	0.7	—	0.45	0.072	44			
	0.7	1.3	—	0.078		39		
	0.7	1.3	0.45	0.042	46		67	68
	0.5	—	—	0.108				
	0.5	—	0.45	0.069	36			
	0.2	—	—	0.085				
	0.2	—	0.45	0.061	35			
	0.2	1.8	—	0.043		50		
	0.2	1.8	0.45	0.024	45		72	76
MgSO ₄ (2 mM) ...	2.0	—	—	0.036				
	2.0	—	0.45	0.008	77			
	1.0	—	—	0.077				
	1.0	—	0.45	0.036	52			
	1.0	1.0	—	0.038		50		
	1.0	1.0	0.45	0.018	54		77	76
	0.7	—	—	0.075				
	0.7	—	0.45	0.032	57			
	0.7	1.3	—	0.038		49		
	0.7	1.3	0.45	0.017	59		73	78
	0.5	—	—	0.072				
	0.5	—	0.45	0.030	58			
	0.5	1.5	—	0.030		58		
	0.5	1.5	0.45	0.014	53		80	83
	0.2	—	—	0.049				
	0.2	—	0.45	0.028	42			
	0.2	1.8	—	0.020		58		
	0.2	1.8	0.45	0.008	58		83	75

Fig. 8. (a) Variation in the activity of mitochondrial pyruvate carboxylase with the concentration of acetyl-CoA in the presence and absence of inhibitors. ●, No additions; ○, 0.145 mM-phosphoenolpyruvate; ▲, 0.60 mM-3':5'-cyclic AMP; △, 1.8 mM-adenosine. (b) Double-reciprocal plot of the results in (a). (c) Hill plot of the results in (a). (d) Plot of $1/v$ versus $1/s^3$ for the results in (a).

concentrations of citrate in excess of 10 mM is explicable in terms of chelation of Mg^{2+} in the medium. Unlike the enzyme from chicken liver (Scrutton, Utter & Mildvan, 1966) and an early report for rat liver (Seubert & Huth, 1965), we have not found inhibition by malate (6 mM) at either high or low concentrations of pyruvate. Similarly, aspartate (1.3 mM), an inhibitor of yeast pyruvate carboxylase (Cazzulo & Stoppani, 1968), has no effect on the rat liver system. Palmitoylcarnitine (0.1 mM) does not alter activity and triethyltin (0.1 mM), an inhibitor of respiration (Aldridge & Rose, 1969), is also without effect.

Inhibition by phosphoenolpyruvate. Although phosphoenolpyruvate chelates Mg^{2+} , its inhibition of pyruvate carboxylase is not due to this property, as addition of excess of magnesium sulphate does not restore the activity of the enzyme. The inhibition induced is mixed with respect to ATP (Fig. 10) but competitive with respect to acetyl-CoA (Fig. 8d). Phosphoenolpyruvate also decreases n for acetyl-CoA, as derived from the Hill plot, from 3.4 to 2.9. This is in contrast with the findings of Utter (1969) who found that the number of sites involved was not altered by the addition of phosphoenolpyruvate. Ling & Keech (1966) found that the

Fig. 9. (a) Inhibition of mitochondrial pyruvate carboxylase by malonyl-CoA: ●, 0.24 mM-acetyl-CoA; ○, 0.05 mM-acetyl-CoA. (b) Dixon plot of the results in (a).

sheep kidney enzyme was not affected by phosphoenolpyruvate. A value of K_i for the rat liver enzyme of 0.55 mM was calculated from Fig. 8(d).

Inhibition by adenosine and adenine nucleotides. Adenosine was found to be a potent inhibitor of rat liver pyruvate carboxylase (Fig. 8). As for the yeast enzyme (Cazzulo & Stoppani, 1969a,b) adenosine is more potent than either AMP, which produces 15% loss of activity when added at 1.82 mM, or ADP, which decreased activity by 28% if added alone or by 15% if added with an equal amount of magnesium sulphate; 1.8 mM-adenosine inhibited the activity by 50%. The same authors found for the yeast enzyme that adenosine was a more effective inhibitor in the acetyl-CoA-activated system, the nucleoside altering the activation produced by the cofactor. From Fig. 8(d) it was estimated that adenosine produced a 40% increment in the K_a towards acetyl-CoA, but the slope of the Hill plot (Fig. 8c) was unaltered. Both K_a and V_{max} were altered by the nucleoside.

Both AMP and low concentrations of ADP (0.2 mM) act as competitive inhibitors of pyruvate carboxylase, with K_i values of 0.76 and 0.25 mM respectively. At 1.84 mM-ADP, in the presence of additional 1.84 mM-magnesium sulphate, the inhibition produced was of a mixed nature (Fig. 10), altering both the K_m and V_{max} of the enzyme. However, the alteration in K_m for ATP by either concentration of ADP is from 0.14 to 0.25 mM. The

inhibition by ADP resembles in all respects that found for the chicken liver enzyme (Scrutton & Utter, 1965) and for the yeast enzyme in the presence of acetyl-CoA (Cazzulo & Stoppani, 1969a,b). In this latter system both AMP and ADP were strictly competitive with respect to ATP in the absence of acetyl-CoA, although AMP was non-competitive when the activator was present, as it competed with acetyl-CoA. The nature of the inhibition produced by AMP in the chicken liver enzyme is somewhat confused, but it is not strictly competitive with either 3'- or 5'-AMP (Scrutton & Utter, 1965).

In contrast with 5'-AMP, 3':5'-cyclic AMP is a non-competitive inhibitor with respect to ATP (Fig. 11), with K_i 1.39 mM. Addition of increasing concentrations of this inhibitor at constant concentrations of ATP produces non-linear Dixon plots (Fig. 11); Hill plots of these results imply that 2 mol of cyclic AMP are attached at 2 mM-ATP, but that at lower concentrations of ATP interactions occur between the molecules of bound inhibitor, n falling from 2.0 at 2 mM-ATP to 1.6 at 0.33 mM-ATP. Cyclic AMP has no effect on the number of molecules of ATP participating in the reaction; further, as no evidence was found of interacting ATP-binding sites, cyclic AMP must act at sites other than those for ATP. When the inhibition produced by cyclic AMP is considered relative to acetyl-CoA, which it is able to replace in yeast (Cooper & Benedict, 1968), it was found to produce similar effects to phosphoenolpyruvate, decreasing n from 3.4 to 2.9, (Fig. 8c) but in this case the inhibition was not strictly competitive.

DISCUSSION

Detailed studies have been published on the possible mechanisms of action of pyruvate carboxylase from chicken liver and sheep kidney (Scrutton & Utter, 1965; Keech & Barritt, 1967). The kinetics of rat liver pyruvate carboxylase as presented in this report differ quantitatively rather than qualitatively from these earlier studies. The requirement of the enzyme for both Mg^{2+} and $MgATP^{2-}$ is consistent with similar findings by Keech & Barritt (1967) but their postulation of interaction between the ions has been criticized by Blair (1969) who re-analysed the data and found it consistent with a model involving random-order binding of Mg^{2+} and $MgATP^{2-}$ without interaction, but with possible additional non-competitive inhibition by ATP^{4-} . We have analysed our pooled results by computer by a similar procedure and find it is also consistent with random binding and non-competitive inhibition by ATP^{4-} . This would be described kinetically by K_a (Mg^{2+}) 0.16–0.28 mM, K_m ($MgATP^{2-}$) 0.13–0.16 mM, K_i

Fig. 10. Activity of mitochondrial pyruvate carboxylase in the presence and absence of inhibitors (8 mM-MgSO₄). (a) ●, No additions; ■, 0.20 mM-ADP; ▲, 0.42 mM-3':5'-cyclic AMP. (b) ●, No addition; □, 1.84 mM-ADP+1.84 mM-MgSO₄ (additional); △, 1.82 mM-AMP; ○, 2.20 mM-phosphoenolpyruvate. (c) Double-reciprocal plots of the inhibition of pyruvate carboxylase by ADP; as the results are derived from both (a) and (b) activity is expressed as a percentage of that at 2 mM-ATP and 8.0 mM-MgSO₄. (d) Double-reciprocal plot of the inhibition of pyruvate carboxylase by 3':5'-cyclic AMP. (e) Double-reciprocal plot of the inhibition of pyruvate carboxylase by AMP. (f) Double-reciprocal plot of the inhibition of pyruvate carboxylase by phosphoenolpyruvate.

Fig. 11. (a) Inhibition of pyruvate carboxylase by 3':5'-cyclic AMP at different concentrations of ATP (8 mM, MgSO_4): $\bullet$, 2.0 mM-ATP; $\blacktriangle$, 0.33 mM-ATP; $\blacksquare$, 0.165 mM-ATP. (b) Dixon plot of the results in (a).

(ATP^{4-}) 1.3–1.4 mM and $V_{\max}$ 135% of the activity at 2 mM-ATP and 8 mM-magnesium sulphate. Cazzulo & Stoppani (1969a,b) concluded that Mg^{2+} activation resulted from the removal of ATP^{4-} whereas excess of Mg^{2+} itself was inhibitory, as we have found. This is also true for phosphofructokinase (Lowry & Passonneau, 1966). Metal-ion inhibition is more pronounced when Mn^{2+} is involved. The postulate of Cazzulo & Stoppani (1969a,b) that the complex ion is bound to the enzyme protein through both its metal and adenosine moieties would also apply to the rat liver enzyme as judged by adenosine inhibition.

The strictly competitive product inhibition by low concentrations of ADP can be ascribed to the reversible replacement of the substrate, presumably as MgADP^- versus MgATP^{2-} . ADP at higher concentrations, producing a mixed type of inhibition relative to ATP, may also bind at the acetyl-CoA site and it is possible that this is due to the unchelated species. AMP, again a competitive inhibitor with respect to ATP, has the same bivalent negative charge as the substrate MgATP^{2-} . The

nature of the non-competitive inhibition of cyclic AMP with respect to ATP is not obvious but may relate to similarity of structure with the adenine moiety of acetyl-CoA. The high concentrations at which it is active suggest that it is unlikely to be of consequence *in vivo* and unrelated to the mechanism by which cyclic AMP stimulates gluconeogenesis (Exton & Park, 1968). As already noted, carboxylated analogues of acetyl-CoA, e.g. malonyl-CoA, inhibit, though homologues can activate (Scrutton & Utter, 1967). The competitive inhibition with respect to acetyl-CoA shown by phosphoenolpyruvate may likewise arise from the proximity of a carboxyl group close to an acetyl-like structure.

The physiological significance of the various inhibitors studied will depend on the actual localization of the enzyme in the cell and on concentrations of both activators and inhibitors in the immediate vicinity. Seubert *et al.* (1968) claim that the enzyme in the rat is bound to the outer mitochondrial membrane, since conditions that solubilize appreciable pyruvate carboxylase solubilize little glutamate dehydrogenase. An earlier study by Wagle (1964) used the property of solubilization of pyruvate carboxylase by 0.25 M-sucrose as a method of extracting the enzyme from the tissue. Henning, Stumpf, Ohly & Seubert (1966) found sufficient extramitochondrial activity of pyruvate carboxylase in both liver and kidney to account for the observed rates of gluconeogenesis and about one-third of the enzyme content in adipose tissue is also in the supernatant fraction (Ballard & Hanson, 1967). In contrast, Marco, Sebastian & Sols (1969) and Böttger, Wieland, Brdiczka & Pette (1969) have reported the enzyme to be exclusively in the mitochondrial matrix. The total liver concentration of acetyl-CoA is about $40 \mu\text{M}$ (Start & Newsholme, 1968) if a water content of 70% is assumed. However, Williamson (1969) has calculated a mitochondrial concentration of acetyl-CoA in the fed rat of up to $250 \mu\text{M}$, with an extramitochondrial concentration of $60 \mu\text{M}$, the intramitochondrial value rising to 1 mM in livers perfused with oleate. At these concentrations pyruvate carboxylase inside the mitochondrion would be fully activated under all conditions. It is noteworthy, however, that the K_a towards acetyl-CoA here appears to be appreciably higher than for the chicken liver enzyme, hence making it somewhat more susceptible to control by changing acetyl-CoA concentrations. Incubation of intact liver mitochondria with octanoate does not stimulate pyruvate carboxylation (Walter, Paetkau & Lardy, 1966), although providing a source of acetyl-CoA and also preventing the removal of this metabolite by inhibition of citrate synthase. On the other hand, a cytosol enzyme is likely to be under variable

activation by acetyl-CoA. Indirect evidence for the existence of extramitochondrial pyruvate carboxylase in the rat comes from the work of Mehlman & Walter (1968). With intact kidney mitochondria they found that the incorporation of pyruvate into organic acids was inhibited by malonyl-CoA, a specific inhibitor of pyruvate carboxylase, as also seen here. As this intermediate cannot cross the mitochondrial membrane, the enzyme must be located on the outer membrane to be subject to inhibition.

Inhibition *in vivo* by adenosine is unlikely, the physiological concentration being far too low. Instances of inhibition of pyruvate carboxylation by ADP in intact mitochondria have been published (Walter *et al.* 1966; Walter & Stucki, 1970), such inhibition being relieved by P_i but not by ATP; this agrees with the mixed type of inhibition by ADP of the purified enzyme. AMP at concentrations similar to those used in this study inhibits the incorporation of lactate into glucose in pigeon liver homogenates (Gevers & Krebs, 1966) but this is thought mainly to be concerned with inhibition of fructose 1,6-diphosphatase.

The significance of inhibition by phosphoenolpyruvate is unclear. Uncoupled mitochondria, in the presence of dinitrophenol, can synthesize measurable amounts of phosphoenolpyruvate (Scholte & Tager, 1965; Bartley & Dean, 1969), despite the fact that the second enzyme in the sequence for the synthesis of this from pyruvate is predominantly in the cytosol. If pyruvate carboxylase is functionally extramitochondrial, feedback inhibition by phosphoenolpyruvate could eliminate the futile recycling of pyruvate through pyruvate kinase, pyruvate carboxylase and phosphoenolpyruvate carboxylase that might otherwise occur.

Of the effects produced by metal ions, Zn^{2+} , although present *in vivo* at concentrations capable of inactivating the enzyme (Long, 1961) is probably in the form of complexes with protein, such as ethanol dehydrogenase. Co^{2+} is unlikely to be of importance, but Mn^{2+} is bound irreversibly to the enzyme (Scrutton *et al.* 1966) and may have a role *in vivo* as an activator. The control of glycolysis and gluconeogenesis by the Mg^{2+}/Ca^{2+} ratio has been considered (Gevers & Krebs, 1966). Ca^{2+} not only inhibits mitochondrial pyruvate carboxylase but also pyruvate kinase in the cytosol; in conditions of high glycolytic activity, entry of Ca^{2+} into the mitochondrion would remove the blockage of pyruvate kinase as well as preventing the resynthesis of phosphoenolpyruvate by pyruvate carboxylase. However, we have shown (Wimhurst & Manchester, 1970a) that fructose 1,6-diphosphatase is inhibited by even lower concentrations of Ca^{2+} than is pyruvate carboxylase; in both enzymes, Mn^{2+} gave some protection against the

inhibition and a balance between the three cations Mg^{2+} , Ca^{2+} and Mn^{2+} may be involved *in vivo*.

Irias, Olmsted & Utter (1969) have shown that chicken liver pyruvate carboxylase has the tetrameric subunit structure typical of an enzyme subject to allosteric control mechanisms. The cold-lability of this enzyme is ascribed to the disaggregation of the subunits, and this is prevented by the presence of the allosteric activator acetyl-CoA, as well as to a lesser extent by various combinations of the substrates (Scrutton & Utter, 1965). The multiplicity of metabolic effectors of pyruvate carboxylase capable of producing a wide range of activities *in vivo* in response to changing tissue requirements without alteration of the content of enzyme protein could explain the relatively few instances of increases found in the total liver content of this enzyme, e.g., on starvation where the cell content of this enzyme remains constant (Wimhurst & Manchester, 1970b).

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