Mechanism of 3-Mercaptopicolinic Acid Inhibition of Hepatic Phosphoenolpyruvate Carboxykinase (GTP)*

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The hypoglycemic agent 3-mercaptopicolinic acid inhibits gluconeogenesis from lactate by isolated, perfused livers from fasted rats and guinea pigs. A 3-mercaptopicolinate concentration of 50 μM caused a sharp decrease in glucose synthesis, with virtually complete inhibition at 100 μM. This inhibitory effect was reversed completely when 3-mercaptopicolinate was removed and the rate of glucose synthesis returned to normal values within 2 min. Oxygen consumption was not altered, even at the highest concentration of inhibitor. Gluconeogenesis from glycerol by guinea pig liver was blocked completely by 100 μM 3-mercaptopicolinate but was inhibited only partially in rat liver. After removal of the inhibitor glucose synthesis returned to levels higher than noted before the addition of this compound. The formation of P-enolpyruvate by isolated guinea pig liver mitochondria metabolizing α-ketoglutarate (State 3) was inhibited markedly by 3-mercaptopicolinate, but malate conversion to P-enolpyruvate was considerably less sensitive to inhibition.

Kinetic studies with purified P-enolpyruvate carboxykinase from rat liver cytosol indicate that 3-mercaptopicolinate is a noncompetitive inhibitor with respect to both oxalacetate and MnGTP2+, and that simultaneous saturation with both substrates does not diminish this inhibition. The inhibitory effects of 3-mercaptopicolinate occur primarily by decreasing the rate of product formation while having relatively minor effects on the apparent Michaelis constants for substrates. Inhibition constants for slope and intercept effects ranged from 2 to 9 μM 3-mercaptopicolinate, and the inhibition patterns were dependent on the concentration of free Mn2+ present. Comparison of the inhibition constants with the observed inhibition of gluconeogenesis in livers perfused with 3-mercaptopicolinate supports the contention that P-enolpyruvate carboxykinase is the site of action of this inhibitor.

The possibility that 3-mercaptopicolinate inhibition occurs by binding either free or bound manganese was eliminated by determination of the dissociation constant of 0.51 mM for the manganese-3-mercaptopicolinate complex. In addition, no tightly bound, slowly exchanging metal was bound to purified enzyme protein. These results suggest that 3-mercaptopicolinate inhibits by the removal of a tightly bound, rapidly exchanging metal ion other than Mn2+.

A recent report by DiTullio et al. (1) demonstrated that 3-mercaptopicolinic acid inhibited both hepatic and renal gluconeogenesis in vitro and was an effective hypoglycemic agent when administered in vivo to fasted or diabetic rats. This compound blocked glucose synthesis from lactate at concentrations as low as 100 μM without altering the rate of urea synthesis, oxygen consumption, or lowering the level of hepatic glycogen. However, gluconeogenesis from fructose was not inhibited by 3-mercaptopicolinic acid suggesting an action of this compound below the triose phosphate level. Preliminary studies by the same investigators suggested an inhibitory effect of 3-mercaptopicolinate on P-enolpyruvate carboxykinase from both rat and guinea pig liver.1

If 3-mercaptopicolinate inhibits hepatic and renal P-enolpyruvate carboxykinase it shares this unique property with the tryptophan metabolite, quinolinic acid (2). Since the two compounds are related structurally, their inhibitory properties suggest a common mechanism. Quinolinate, however, is effective at concentrations in the millimolar range, whereas 3-mercaptopicolinate inhibits glucose synthesis in the range of 50 to 100 μM. Snoke et al. (2) have suggested, from studies with rat liver cytosol containing P-enolpyruvate carboxykinase, that quinolinate inhibits this enzyme by chelating Fe3+ normally

bound to the enzyme. These studies were necessarily indirect, since the authors contend that Fe** dissociates from the enzyme when it is diluted for assay. In the same paper, Snake et al. investigated a number of tryptophan metabolites, analogues, and known metal chelators for their inhibitory effect on Fe**-activated P-enolpyruvate carboxykinase and found that quinoline was the most potent inhibitor, causing a 50% decrease in enzyme activity at a concentration of 2 mM. The mechanism of this quinolinate effect was studied by McDaniel et al. (3), using partially purified P-enolpyruvate carboxykinase from rat liver cytosol. Quinoline was noncompetitive with oxaloacetate (K = 500 μM) but its ferrous form was reported as being competitive against oxaloacetate with a K1 - 100 μM. Thus, quinoline does inhibit P-enolpyruvate carboxykinase, but at concentrations far in excess of the levels 3-mercaptopicolinate required for the same degree of inhibition.

In view of its potent hypoglycemic effect in vivo as well as its ability to inhibit hepatic glucoseogenesis (1), 3-mercaptopicolinic acid may provide a valuable tool for studies of the regulation of hepatic glucose synthesis. In the present report we have extended the initial observations of Ditullio et al. (1) to include a detailed analysis of the mechanism of action of this hypoglycemic agent. Our results indicate that 3-mercaptopicolinate can inhibit specifically P-enolpyruvate carboxykinase from both rat and guinea pig liver and that the K1 for the rat liver cytosol enzyme is approximately 3 μM.

**EXPERIMENTAL PROCEDURE**

*Chemicals*—L (+) Lactic acid, ammonium free urease type VI (EC 3.5.1.5), glucose oxidase (EC 1.1.1.34), and horseradish peroxidase (EC 1.1.1.17) were from Sigma. Glycerol was purchased from Baker. Malate, α-ketoglutarate, and Hepe* were obtained from Calbiochem. 3-Mercaptopicolinic acid was generously provided by Dr. Harry Saunders, from Smith, Kline and French, Inc., Philadelphia, Pa. Rat liver cytosol P-enolpyruvate carboxykinase (EC 4.1.1.32) with a specific activity of 10 units/mg of protein (assayed in the direction of CO2 fixation, see below), was purified by the method of Ballard and Hanson (4). NAD+, NADH, ADP, pyruvate kinase (EC 2.7.1.40), malate dehydrogenase (EC 1.1.1.37), and lactate dehydrogenase (EC 1.1.1.37) were obtained from Boehringer Mannheim Corp.

*Animals*—Male guinea pigs (200 to 250 g) of the Hartley strain were fed ad libitum on Wayne guinea pig chow and greens. Male Sprague-Dawley rats (150 to 200 g) from Charles River were also fed ad libitum on Purina Chow.

*Metabolic Studies*—After a 24-hour fast for rats or a 48-hour fast for guinea pigs, the animals were anesthetized and the livers perfused as described previously with a non-recirculating system (5). The glucose concentration in the perfusate was determined by the glucose oxidase-peroxidase method (6) in a Technicon autoanalyzer. Liver mitochondria were isolated from fed guinea pigs by differential centrifugation (7). Mitochondria (approximately 12 mg of protein) were incubated in 3 ml of 160 mM sucrose, 2 mM Hepes, pH 7.5, 6.6 mM MgCl2, 25 mM ADP, and 2 mM malate or succinate for 10 min at 30°C. Incubations were stopped with chilled perchloric acid (final concentration 6% w/v) and P-enolpyruvate formation was measured spectrophotometrically after neutralization of the extracts (8). Mitochondrial protein concentration was determined by ultraviolet absorption (9).

*Phosphoenolpyruvate Carboxykinase Activity Measurements*—Enzyme activity was measured in two ways. Using crude extracts (guinea pig liver mitochondria and cytosol or rat liver cytosol), P-enolpyruvate activity was determined by the carboxylation reaction described by Chang and Lane (10) as modified by Ballard and Hanson (4). Kinetic analysis was performed with P-enolpyruvate carboxykinase from rat liver cytosol using a spectrophotometric method (11). In this assay the formation of P-enolpyruvate was measured by equilibrium displacement in a system in which oxaloacetate was generated from malate by malate dehydrogenase. The 2 ml reaction mixture contained 50 mM Tris-HCl, pH 8.0, 0.75 mM MnCl2, 1 mM NAD+, malate dehydrogenase (8 units), purified P-enolpyruvate carboxykinase (0.015 unit), and 20 mM malate except in experiments where various concentrations of oxaloacetate were generated from malate. The oxaloacetate formed from malate dehydrogenase was determined spectrophotometrically by measuring the NADH generated in the reaction after equilibration at 37°C for 4 min. GTP was then added to start the reaction. Initial rates were taken from the linear portion of the recording. Doubling the malate dehydrogenase concentration had no effect on initial rate measurements thereby assuring that malate dehydrogenase was not the limiting enzyme.

*Analysis of Data*—Double reciprocal plots of initial velocity as a function of substrate concentration were analyzed according to the methods outlined by Cleland (12). Data were fitted by computer to Equations 1, 2, 3, or 4 when graphical analysis of the results confirmed to sequential substrate addition, linear noncompetitive inhibition, slope linear-intercept hyperbolic noncompetitive inhibitor, or a parabolic function of reciprocal substrate concentration, respectively (13).

**FITS MADE TO EQUATION 4 SHOWED DEVIATIONS FROM THE EXPERIMENTAL POINTS AT HIGHER SUBSTRATE CONCENTRATIONS WHEN RECIPROCAL INITIAL VELOCITIES WERE UNWEIGHTED OR WERE WEIGHTED BY A FACTOR OF 1/V, GOOD CORRESPONDENCE BETWEEN EXPERIMENTAL POINT AND THEORETICAL CURVES WERE OBTAINED WITH A WEIGHTING FACTOR OF 1/V, WHICH WAS USED FOR ANALYZING THE DATA SHOWN IN FIG. 7. THE VALUES OF KINETIC CONSTANTS OBTAINED FROM FITTING DATA TO EQUATIONS 1 TO 4 WERE USED TO DRAW THE LINES OF BEST FIT TO THE EXPERIMENTAL POINTS.**

**Electron Paramagnetic Resonance Measurements**—The manganese paramagnetic resonance measurements were kindly made by Dr. George Reed at the University of Pennsylvania using a Varian model E-3 electron spin resonance spectrometer. Measurements were made on 50-ml samples at 37°C in 0.05 M Hepes buffer, pH 8.0, containing a total of 0.1 M MnCl2. The manganese was titrated with a 1.0 mM 3-mercaptopicolinate solution containing 0.1 mM MnCl2 and 0.05 M Tris buffer, pH 8.0. The reaction mixture was assumed to be proportional to free manganese concentration. The dissociation constant was calculated from the observed decrease in peak height when approximately 60% of the signal was abolished by the added 3-mercaptopicolinate.

**Metal Analysis**—Determination of metals was done by atomic absorption spectroscopy using an Aztec Atomic Analyzer Mark II spectrophotometer equipped with a Varian Techtron model 63 carbon rod atomizer. Enzyme samples were prepared by resuspending 1 mg of purified P-enolpyruvate carboxykinase in ammonium sulfate, and 2 ml of 0.05 M tris(hydroxymethyl)-HCl, pH 8.0, containing 1 mM EDTA and 0.1 mM dithiothreitol. This sample was passed through a column (0.63 x 22 cm) of Sephadex G-100 equilibrated with the same buffer. Fractions of 0.15 ml were collected and fractions containing the purified P-enolpyruvate carboxykinase ammonium sulfate precipitate were pooled. Metal ions were determined spectrophotometrically after neutralization of the extracts (9). Mitochondrial protein concentration was determined by ultraviolet absorption (10).

The abbreviation used is: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

38 Mercaptopicolinate Inhibition of P-enolpyruvate Carboxykinase

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Results

The effect of 3-mercaptopicolinate on glucose synthesis and oxygen consumption by isolated perfused rat and guinea pig livers is shown in Fig. 1. Increasing concentrations of the inhibitor from 25 to 100 μM caused a virtual cessation of gluconeogenesis from lactate with no effect on hepatic oxygen consumption. When 3-mercaptopicolinate infusion was terminated at 80 min, the rate of hepatic gluconeogenesis returned immediately to control levels indicating that the inhibitory effect of this compound was reversed rapidly. Gluconeogenesis from glycerol, on the other hand, was less sensitive to 3-mercaptopicolinate (Fig. 2). Rat liver was only marginally sensitive to the inhibitor at concentrations (100 μM) which were sufficient to totally abolish gluconeogenesis from lactate. The conversion of glycerol to glucose was lower in guinea pig liver and 100 μM 3-mercaptopicolinate completely depressed glucose synthesis. The reason for this difference in the effect of 3-mercaptopicolinate on gluconeogenesis from glycerol by rat as compared to guinea pig liver is not clear. However, there was no significant inhibition of oxygen consumption by livers from either species with glycerol as substrate, even at the highest 3-mercaptopicolinate concentration.

Since the synthesis of mitochondrial P-enolpyruvate is involved directly in gluconeogenesis from lactate in guinea pig liver (15), we tested the effect of increasing concentrations of 3-mercaptopicolinate on P-enolpyruvate synthesis from malate or from α-ketoglutarate (Fig. 3). P-enolpyruvate formation from α-ketoglutarate was sensitive to inhibition, and at 100 μM 3-mercaptopicolinate its synthesis was inhibited completely. Malate conversion to P-enolpyruvate was also decreased but only at higher inhibitor levels. Also, 3-mercaptopicolinate did not alter oxygen consumption in these isolated mitochondria nor did it alter the respiratory control ratio of about 7, normally observed with guinea pig liver mitochondria.

DiTullio et al. (1) have suggested that 3-mercaptopicolinate inhibits P-enolpyruvate carboxykinase. This suggestion is
consistent with the mitochondrial studies described above and led us to study the effect of this inhibitor on the enzyme from rat liver cytosol and from guinea pig liver mitochondria and cytosol. When the activity of purified P-enolpyruvate carboxykinase from the cytosol of rat liver or the unpurified enzyme from both mitochondria and cytosol of guinea pig liver was assayed in the direction of "CO₂ fixation the observed K, for 3-mercaptopicolinate was 1 mM (data not shown). A similar observation was made by Kostos and co-workers using the cytosol of rat liver. For a more detailed analysis of the effect of 3-mercaptopicolinate on P-enolpyruvate carboxykinase, we measured the effect of the inhibitor on the purified enzyme from rat liver cytosol in the direction of P-enolpyruvate formation and noted a striking inhibition of the enzyme by 3-mercaptopicolinate. As noted in detail below, a K, of 2.8 μM was observed using this assay. We have no explanation for this difference at the present time but an investigation of its cause is planned.

### Initial Velocity Patterns for P-enolpyruvate Carboxykinase

In the absence of inhibitors, double reciprocal initial velocity data with MnGTP⁻ as the variable substrate and oxalacetate as the fixed variable gave a pattern of intersecting lines (Fig. 4) which suggest a sequential addition of substrates to the enzyme and the formation of a ternary complex before products are released. Analysis of the data according to Equation 1 gave the values for Kₐ, Kₐ, and Kₐ listed in Table I. These results indicate that both substrates combine well with the enzyme since the apparent Michaelis, or dissociation constants, or both, for oxalacetate and MnGTP⁻ are in the range from 5 to 15 μM. It is interesting to compare the apparent Kₐ values for oxalacetate obtained from this study (5 to 19 μM, see Table I) with those obtained by other investigators. These values vary from approximately 1 to 4000 μM for both cytosolic and mitochondrial enzymes (16-18). These observed differences may be due to the use of nonoptimal divalent cation concentrations, to erroneous oxalacetate concentrations arising from the labile nature of oxalacetate, or to the severe substrate inhibition caused by excessive concentrations of malate in coupled assays (19). These problems were avoided since oxalacetate was generated continually from noninhibitory concentrations of malate by the malate dehydrogenase reaction (see "Experimental Procedure"), and total manganese was usually maintained equal to or in excess of the total GTP concentrations but below levels which have been found to be inhibitory. Under these conditions, most of the GTP is present as the MnGTP⁻⁻ complex, which has been suggested as the substrate for P-enolpyruvate carboxykinase (20).

### Inhibition of P-enolpyruvate Carboxykinase by 3-Mercaptopicolinate

Increasing fixed levels of 3-mercaptopicolinate caused both slope and intercept changes in double reciprocal plots as a function of 1/oxalacetate (Fig. 5). The data gave good fits to the equation for linear noncompetitive inhibition (Equation 2) and the kinetic constants are shown in Table I. In this experiment MnCl₂ and GTP were fixed at 0.75 mM. Under these conditions the free Mn²⁺ and GTP concentrations will be approximately 80 μM. In order to confirm that the slope and intercept values are linear functions of 3-mercaptopicolinate, additional experiments were done using a wider range of inhibitor concentration. Each line was fitted independently by the unweighted least squares method and the resulting slope and intercept values were plotted. These results, shown in Fig. 5 (inset), confirm that slope and intercept are linear functions of inhibitor concentration.

Similar inhibition experiments with MnGTP⁻⁻ as the variable substrate, and with free Mn²⁺ being maintained from 0.4 to 0.7 mM also gave noncompetitive inhibition; however the ordinate intercepts were a hyperbolic rather than a linear function of 3-mercaptopicolinate concentration. Kinetic constants, obtained by fitting the data of Fig. 6 to Equation 3, are listed in Table I. The hyperbolic ordinate intercepts indicate that when both MnGTP⁻⁻ and inhibitor are saturating, only partial inhibition will occur. The degree of inhibition

### Table I

| Kinetic parameters for substrate and 3-mercaptopicolinate interaction with P-enolpyruvate carboxykinase |
|---|---|---|
| **Substrate or inhibitor** | **Kinetic constant** | **Value** | **Experiment** |
| Oxalacetate | Kₛ | 9 ± 1 | Fig. 4 |
| | Kₐ | 18 ± 3 | Fig. 5 |
| | Kₛₕ/Kₐ | 6 ± 4 | Fig. 4 |
| | Kₛ | 16 ± 5 | Fig. 4 |
| | Kₐ | 22 ± 2 | Fig. 6 |
| | Kₛ | 8 ± 5 | Fig. 4 |
| | Kₛₙ | 7 ± 2 | Fig. 5 |
| | Kₛ | 9 ± 3 | Fig. 6 |
| | Kₛₕ | 3.5 ± 0.8 | Fig. 5 |
| | Kₛₙ | 1.5 ± 0.2 | Fig. 6 |
| | Kₐ | 9 ± 1 | Fig. 6 |
| | Kₛ | 2.0 ± 0.2 | Fig. 7 |
at saturating inhibitor concentration can be determined from the ratio of $K_{o}$ to $K_{m}$. Under these conditions, saturation with 3-mercaptopicolinate will result in approximately 70% inhibition at saturating MnGTP$^{2-}$. The concentration of 3-mercaptopicolinate required to cause one-half of the change in ordinate intercept is given by $K_{o}$ and was determined to be 1.5 μM (Fig. 6, inset). In contrast to the hyperbolic intercept values, the slope values appear to be a linear function of 3-mercaptopicolinate. However, under these experimental conditions, the slope effects are smaller and more subject to error than the intercept effects and the possibility of slope-hyperbolic effects cannot be definitely ruled out from these results alone. The concentration of 3-mercaptopicolinate required to double the slope is given by $K_{m}$ and was determined to be 9 μM. These results indicate that the major effect of 3-mercaptopicolinate is to reduce the catalytic capacity as concentrations of inhibitor are increased over the range of this experiment.

When the experiment shown in Fig. 6 was repeated under conditions where the free Mn$^{2+}$ was maintained below 40 μM, linear noncompetitive inhibition resulted. Under these conditions the $K_{m}$ and $K_{o}$ values of 9 ± 4 and 7 ± 1 μM, respectively, for 3-mercaptopicolinate were approximately the same as those obtained when free Mn$^{2+}$ was present at 0.4 to 0.7 mM. Thus, the concentration of free Mn$^{2+}$ affects the type of inhibition pattern obtained, but does not affect the concentration of inhibitor needed for slope and intercept effects. These results suggest that relatively high concentrations of free Mn$^{2+}$ must be present to prevent complete inhibition by 3-mercaptopicolinate.

Since 3-mercaptopicolinate was noncompetitive against both substrates, the results indicate that it does not bind at either substrate site, and therefore may act as an inhibitor at saturating concentrations of both substrates. In order to confirm this supposition, the effect of 3-mercaptopicolinate on initial velocity was studied under conditions where both substrates were varied in constant ratio. This experimental approach is useful for several reasons. First, in the absence of inhibitor, the double reciprocal plot of such data should be parabolic if the reaction is sequential (21). Secondly, ordinate intercept values will increase only if 3-mercaptopicolinate is capable of inhibiting the reaction when the enzyme is fully saturated with substrates. Thirdly, if intercept differences are observed, the inhibition constant describing the interaction of inhibitor with substrate-saturated enzyme can be obtained from the ordinate intercept replot. Finally, these constants can be compared with those obtained from the inhibition experiments in Figs. 5 and 6 to determine whether the inhibitor acts equally well when the enzyme is partially or fully saturated with substrates. The results of such an experiment are shown in Fig. 7. The data gave good fits to Equation 4 (see "Experimental Procedure") when each line was fitted independently. The ordinate intercept values obtained from fitting the data were replotted as a function of the inhibitor (Fig. 7, inset) and approximate a linear function of its concentration over the experimental range. The replot should theoretically be hyperbolic, since the free Mn$^{2+}$ concentration was maintained above 0.4 mM. A more definitive analysis of this data was
Mercaptocollinate Inhibition of P-enolpyruvate Carboxykinase

Mercaptocollinate Inhibition of P-enolpyruvate Carboxykinase

The peak tube from the Sephadex G-100 column contained approximately 0.33 mg/ml of P-enolpyruvate carboxykinase. Samples of 5 µl were analyzed for metal content as described under “Experimental Procedure.”

<table>
<thead>
<tr>
<th>Metal measured</th>
<th>Metal content</th>
<th>Molar ratio, enzyme/metal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron</td>
<td>15</td>
<td>&lt;0.08</td>
</tr>
<tr>
<td>Manganese</td>
<td>6</td>
<td>&lt;0.023</td>
</tr>
<tr>
<td>Magnesium</td>
<td>5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Calcium</td>
<td>10</td>
<td>&lt;0.08</td>
</tr>
<tr>
<td>Copper</td>
<td>2</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Zinc</td>
<td>23</td>
<td>&lt;0.09</td>
</tr>
<tr>
<td>Cobalt</td>
<td>18</td>
<td>&lt;0.07</td>
</tr>
</tbody>
</table>

DISCUSSION

The results of this study indicate that mercaptocollinate, at low concentrations (50 µM) can block hepatic gluconeogenesis, presumably by inhibiting P-enolpyruvate carboxykinase. This compound is the first inhibitor described which causes hypoglycemia and also inhibits P-enolpyruvate carboxykinase. In verification of the original report by DiTullio et al. (1), we noted that mercaptocollinate, administered orally to rats at a concentration of 15 mg/100 g body weight caused a fall in the blood glucose concentration from 4.2 mM to about 1 mM in only 3 hours. This compound also effectively inhibits hepatic gluconeogenesis in perfused rat and guinea pig livers, with 100 µM causing a virtually complete cessation of glucose synthesis from lactate. Quinolinate has also been used to block hepatic gluconeogenesis by inhibiting P-enolpyruvate carboxykinase. This compound does not cause hypoglycemia. Silling and his co-workers (22) reported that quinolinate inhibits gluconeogenesis from lactate in perfused rat liver but causes only a 50% inhibition in guinea pig liver and is without effect on glucose synthesis by perfused pigeon liver. Since the latter two species have 50 and 95%, respectively, of their hepatic P-enolpyruvate carboxykinase in the mitochondria, it is probable that quinolinate does not inhibit this enzyme. In contrast, mercaptocollinate will block gluconeogenesis completely in perfused guinea pig liver and also causes partial inhibition of the synthesis of P-enolpyruvate from either malate or α-keto glutarate (Fig. 3). The reason for the differential sensitivity of P-enolpyruvate synthesis from α-keto glutarate as compared to malate in the presence of 3-mercaptopicolinate is not understood. It is possible that the level of oxalacetate generated within the mitochondria is greater with malate than with α-keto glutarate. A higher concentration of mitochondrial oxal-
acetate would result in a less effective inhibition of P-enolpyruvate carboxykinase (see Fig. 5) by 3-mercaptopicolinate.

If 3-mercaptopicolinate inhibits gluconeogenesis solely by acting at P-enolpyruvate carboxykinase, it is difficult to explain its complete suppression of glucose synthesis from glycerol in perfused guinea pig liver. This effect appears species-dependent since we noted only a marginal inhibition of glucose synthesis from glycerol by rat liver. DiTullio et al. (1) reported that 3-mercaptopicolinate did not depress the rate of gluconeogenesis from fructose so that any effect of this inhibitor at a step other than P-enolpyruvate carboxykinase must be below the triose phosphate level. Alternatively, some portion of the glycerol destined for conversion to glucose may be recycled through the pathway involving mitochondrial P-enolpyruvate carboxykinase. ATP is required for the initial phosphorylation of glycerol via glycerol kinase and since no other substrate was perfused with glycerol in these experiments, some oxidation of glycerol in the citric acid cycle is probable. Further experiments are required to establish the pathways of carbon flow with glycerol as the sole gluconeogenic substrate, but the results do indicate the usefulness of a specific inhibition of P-enolpyruvate carboxykinase in elucidating potential sites of regulation of gluconeogenesis.

Considering its importance as a major gluconeogenic enzyme, the mechanism of action of P-enolpyruvate carboxykinase is poorly understood. Values for the $K_m$ of oxalacetate for the enzyme vary from 4 mM (17) to 1.5 [$\mu$M] (16) depending on the source of the enzyme and the method of assay. Our problem, pointed out by Ballard (19), is the rapid breakdown of oxalacetate added for assay, particularly when kinetic studies are carried out with tissue extracts. Measurements of the $K_m$ for oxalacetate reported in this paper used oxalacetate maintained in equilibrium with malate by the malate dehydrogenase reaction. Using a highly purified P-enolpyruvate carboxykinase (specific activity of 10 units/mg of protein) we find a $K_m$ of 9 [$\mu$M] for oxalacetate and a $K_m$ of 16 [$\mu$M] for MnGTP$^{2-}$.

The concentration of oxalacetate in perfused rat liver cytosol has been estimated to be 5 to 10 [$\mu$M] (23), a value close to the $K_m$ of oxalacetate of P-enolpyruvate carboxykinase noted in this study. However, estimates of the GTP concentration in the whole liver by Clifford et al. (24) give values of 600 [$\mu$M], and those of Chance et al., 100 [$\mu$M] (25). If GTP were distributed evenly between the mitochondria and cytosol, P-enolpyruvate carboxykinase would be nearly saturated with respect to this nucleotide. This finding sheds some doubts on a possible regulatory role of GTP in P-enolpyruvate formation, suggested from studies with isolated guinea pig liver mitochondria (26, 27).

Kinetic studies of the inhibition by 3-mercaptopicolinate have also provided information concerning the role of metal ions in the P-enolpyruvate carboxykinase reaction. Previous investigators have demonstrated that divalent cation-chelating agents such as quinolinol and picolinate cause inhibition of the impure enzyme (2). The mechanism for such inhibition has remained obscure, since the role of metal ions in the enzyme has not been established unequivocally, although Miller et al. (28) showed that 1 molecule of manganese could bind to free enzyme with a dissociation constant of approximately 40 [$\mu$M]. Although 3-mercaptopicolinate does chelate manganese, inhibition of P-enolpyruvate carboxykinase is unlikely to occur by such chelation since the total manganese was always present in a large molar excess relative to the inhibitor and the dissociation constant for the manganese-chelator complex is too high to permit significant depletion of either free or enzyme-bound manganese. These observations, together with the results of Snoke et al. (2), which showed that preincubation of the enzyme with a number of divalent cations increased initial reaction rates, suggest that a metal-enzyme complex is necessary for effective catalysis. The apparent absence of tightly bound, slowly dissociating metal makes it likely that one or more rapidly exchanging metal ions other than manganese are involved.

The reaction mechanism shown in Scheme I is consistent with the kinetics of 3-mercaptopicolinate inhibition as well as the observations of previous workers. In this scheme, $E$ - enzyme, $3$-MPA = 3-mercaptopicolinate, and $K_1$ and $K_2$ represent the rate of product formation from the enzyme-M, and enzyme-manganese complexes, respectively. The free enzyme is proposed to bind either manganese or another metal ion, designated as M, to satisfy the requirement for enzyme-bound metal. The concentration of metal ions such as Fe$^{2+}$, Co$^{2+}$, Zn$^{2+}$, and others normally contaminating reaction mixtures are assumed to be sufficient to provide quantities of M, in excess of the enzyme concentrations used in enzyme assays (usually $4 \times 10^{-9}$ M). The reports of Snoke et al. (2) have suggested that M, may be ferrous iron in vivo, however, a careful inspection of the data suggest that cobalt could also have been effective under the conditions of that study. In addition to M,, a metal is required in formation of the metal-GTP complex. This requirement is met by Mn$^{2+}$ or other ions can fulfill this requirement, since small amounts of Mn$^{2+}$ or other metal ions can activate the enzyme when the GTP is almost all present as the MgGTP$^{2-}$ complex (29).

Scheme I is consistent with the finding that 3-mercaptopicolinate causes linear noncompetitive inhibition in the absence of free Mn$^{2+}$, but gives intercept-hyperbolic noncompetitive inhibition when free Mn$^{2+}$ is present at concentrations in excess of the dissociation constant previously reported for the enzyme-manganese complex. If the chelating agent were to act by forming an inactive dead-end enzyme-3-mercaptopicolinate complex, the inhibition patterns with respect to oxalacetate and MnGTP$^{2-}$ would both be slope and intercept-linear patterns. However, if inhibition occurred by the formation of an enzyme-3-mercaptopicolinate complex which retained partial activity, inhibition patterns with both oxalacetate and MnGTP$^{2-}$ would be expected to give intercept-hyperbolic patterns. The conversion of intercept-hyperbolic to intercept-linear inhibition by 3-mercaptopicolinate with variable MnGTP$^{2-}$ at high and low free Mn$^{2+}$ concentrations, respectively, provides support for the hypothesis that manganese can fulfill the role of the enzyme-activating metal. This activation is shown by the lower reaction sequence in Scheme I. Under the assay conditions which were used, the apparent $k_1/k_2$ ratio is approximately 4, indicating that manganese is less efficient in activating the enzyme than M, the non-manganese ion.

\[ \text{Mn, M, 3-MPA} \]

\[ 3\text{-MPA} \]

\[ E = M \rightarrow M, E \rightarrow OAA, MnGTP^{2-} \]

\[ M, E, OAA, MnGTP \]

\[ \text{Products} \]

\[ M, E, OAA, MnGTP \]

\[ Mn \rightarrow OAA, MnGTP^{2-} \]

\[ Mn, E \]

\[ \text{Products} \]

\[ Mn, E \]

\[ 3\text{-MPA} \]

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\[ Mn, E \]

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\[ E = M \rightarrow M, E \rightarrow OAA, MnGTP^{2-} \]

\[ M, E, OAA, MnGTP \]

\[ \text{Products} \]

\[ M, E, OAA, MnGTP \]

\[ Mn \rightarrow OAA, MnGTP^{2-} \]

\[ Mn, E \]

\[ \text{Products} \]

\[ Mn, E \]
value is only an apparent constant, since the true \( k_1 \) and \( k_2 \) ratio must be determined as \( V_{max} \) values at saturating but noninhibitory concentrations of \( M_1 \) and \( Mn^{++} \), respectively. Such experiments are complicated by the binding of both metal ions to enzyme and GTP thereby giving a mixture of four metal-ligand complexes, all of which may have unique kinetic parameters for interaction with the other components of the system. Further work is needed to clarify the interaction of these metal ions with P-enolpyruvate carboxykinase.

The order of substrate addition to the enzyme is not indicated in Scheme I, however initial velocity patterns indicate that the addition is sequential rather than ping-pong. More experiments are needed to determine the order of \( MnGTP^2- \) and oxalacetate addition since literature reports are conflicting (17, 20) and are primarily concerned with the order of P-enolpyruvate, nucleoside diphosphate, and \( CO_2 \) addition.

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