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# THE MECHANISM OF INHIBITION OF PHOSPHOENOLPYRUVATE CARBOXYLASE BY QUINOLINIC ACID

H. G. McDANIEL, W. J. REDDY AND B. R. BOSHELL

*V A Hospital and the Department of Medicine, University of Alabama School of Medicine, Birmingham, Ala. 35233 (U.S.A.)*

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SUMMARY

The effect of quinolinic acid and its ferrous and manganous derivatives on phosphoenolpyruvate (PEP) carboxylase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) from rat liver cytoplasm is dependent upon the concentration of oxaloacetate in the assay system. At limiting levels of oxaloacetate all three compounds are inhibitory. At saturating levels of oxaloacetate the concentration of quinolinic acid and ferrous quinolinate required to inhibit PEP formation is increased and manganous quinolinate actually stimulates the reaction. Reciprocal plots show that ferrous quinolinate is a competitive inhibitor and manganous quinolinate produces a mixed type of inhibition. Removal of one of the carboxyl groups of quinolinic acid so that it no longer has the same dicarboxylic structure as oxaloacetate markedly decreases its ability to inhibit PEP carboxylase. The calculated $K_i$ for ferrous quinolinate is $1 \cdot 10^{-4}$ M. The intraperitoneal injection of 10 mg of L-tryptophan/100 g of body weight produces a quinolinic acid concentration of $3.7 \cdot 10^{-4}$ M and a 5-fold rise in the level of malate in the liver indicating the cytoplasmic PEP carboxylase is blocked. Concentrations of quinolinic acid in the liver below $1 \cdot 10^{-4}$ M may also influence this reaction through the chelation of ferrous ions as suggested by Lardy.

## INTRODUCTION

Large amounts of L-tryptophan will produce hypoglycemia in fasted rats[1]. This hypoglycemia is associated with a decrease in the level of phosphoenolpyruvate (PEP) in the liver and a marked increase in the level of oxaloacetate and its precursors in gluconeogenesis: malate, aspartate and citrate[2]. Tryptophan metabolites in the nicotinic acid pathway through quinolinic acid all produce this alteration in liver metabolites suggesting that quinolinic acid is responsible for the inhibition of gluconeogenesis at this level[3]. Lardy has shown that ferrous quinolinate will inhibit PEP carboxylase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32)

Abbreviation: PEP, phosphoenolpyruvate.

*in vitro* and has proposed that this compound inhibits the PEP carboxylase reaction *in vivo*[4]. We have investigated the mechanism by which quinolinic acid and its metal chelates inhibit the cytoplasmic PEP carboxylase and have calculated a $K_i$ for ferrous quinolinate. These *in vitro* studies have been correlated with the effect of a given dose of tryptophan on the carboxylase step *in vivo* and the level of quinolinic acid achieved in the liver from this dose. The final discussion concerns whether the inhibition of PEP carboxylase by quinolinic acid is of physiological or of pharmacological significance.

## METHODS

PEP carboxylase was prepared from the liver of 150–200 g male Sprague–Dawley rats (Vinemont Farms) which had been fasted overnight[5]. They were killed with a blow to the head and the liver removed and homogenized in 0.25 M sucrose containing 0.05 M Tris–HCl buffer (pH 7.4). After the homogenate was centrifuged at 40 000 × *g* for 1 h, the precipitate was discarded and the supernatant brought to 40% saturation with solid ammonium sulfate (calculated at 25 °C). This solution was then centrifuged, the precipitate again discarded and the supernatant brought to 70% saturation with solid ammonium sulfate. After adjusting the pH to 7.0 with solid Tris the enzyme preparation was stored at 2 °C. This fraction had a specific activity of 0.1 $\mu$mole·min$^{-1}$·mg$^{-1}$ and was stable on storage at 2 °C for 6 weeks. PEP carboxylase was assayed by measuring the formation of PEP with pyruvate kinase and lactate dehydrogenase according to the method of Seubert and Huth[6].

Ferrous and manganous quinolinate were made by adding ferrous chloride or manganous chloride to a solution of quinolinic acid. The chemicals and enzymes used were all purchased from commercial sources. Oxaloacetate was made up fresh each week. Quinolinic acid was measured in the liver of fasted animals 3 h after a given dose of tryptophan by the procedure of Waisman and Elvehjem[7]. Malate was measured by the standard enzymatic assay on $HClO_4$ extracts of livers which had been frozen within 30 s after their removal[2].

The spontaneous decarboxylation of oxaloacetate to pyruvate, under the experimental conditions used to assay PEP carboxylase activity, was measured with lactate dehydrogenase using a Gilford recording spectrophotometer[2].

## RESULTS

When the formation of PEP from oxaloacetate is assayed in the presence of a saturating level of oxaloacetate there is no inhibition by quinolinate at a concentration of 1 mM (Table I). Ferrous quinolinate at the same concentration is slightly inhibitory and manganous quinolinate actually stimulates the reaction. These compounds are much more effective inhibitors of this reaction at a limiting level of oxaloacetate (Table II). Even manganous quinolinate which is stimulatory at a saturating level of oxaloacetate produces a marked degree of inhibition. In this assay system the $K_m$ for oxaloacetate was 0.5 mM so that the concentration of oxaloacetate used in Table II was limiting. (This is higher than the actual $K_m$ because no adjustment was made for the spontaneous decarboxylation of oxaloacetate to pyruvate). The four carbon dicarboxylic part of quinolinic acid which is structurally very similar to *cis*-

TABLE I

THE EFFECT OF QUINOLINIC ACID AND RELATED COMPOUNDS ON PEP FORMATION FROM OXALOACETATE AT A SATURATING LEVEL OF OXALOACETATE

The incubation mixture containing Tris–HCl, 100 mM (pH 8.0); ITP, 6 mM; 2-Mercaptoethanol, 0.9 mM; $MgCl_2$, 20 mM; KCl, 15 mM; KF, 15 mM; oxaloacetate, 5 mM and 1 mg of the enzyme preparation in a total volume of 1 ml was incubated at 30 °C for 5 min. The reaction was stopped by the addition of 10–15 mg of sodium borohydride which converted the remaining oxaloacetate to malate. After 2 min the excess sodium borohydride was oxidized by the addition of 0.3 ml of 6% perchloric acid. Following neutralization with KOH to pH 7.0 and centrifugation, PEP was assayed spectrophotometrically with pyruvate kinase and lactate dehydrogenase.

| *Compound added* | *Concentration (mM)* | *Alteration in activity (% change from the basal value)* |
|---|---|---|
| None | – | 0 |
| Quinolinic acid | 1 | 0 |
| Manganous quinolinate | 1 | +40 |
| Ferrous quinolinate | 1 | −22 |

TABLE II

THE EFFECT OF QUINOLINIC ACID AND RELATED COMPOUNDS ON PEP FORMATION FROM OXALOACETATE AT A LIMITING LEVEL OF OXALOACETATE

The incubation mixture was the same as in Table I except for the concentration of oxaloacetate which was 0.8 mM.

| *Compound added* | *Concentration (mM)* | *Inhibition (%)* |
|---|---|---|
| Quinolinic acid | 1 | −70 |
| Manganous quinolinate | 1 | −72 |
| Ferrous quinolinate | 1 | −76 |
| Picolinic acid | 1 | −29 |
| Nicotinic acid | 1 | 0 |

oxaloacetate (Fig. 1) is important for the inhibitory properties of quinolinic acid. Removal of the $\beta$-carboxyl group produces picolinic acid which is much less inhibitory (Table II) and removal of the $\alpha$-carboxyl group results in nicotinic acid which does not have any inhibitory effect. Changing the concentration of ITP or of magnesium did

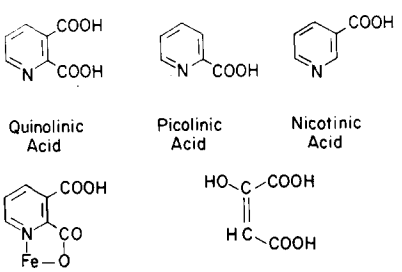
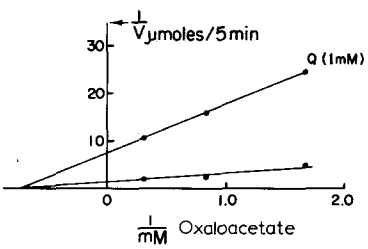



Fig. 1. Although quinolinic acid more nearly resembles the *cis*-enol form of oxalacetate the keto form of oxalacetate has been shown to be the substrate for phosphoenolpyruvate carboxylase (from chicken liver)[19]. Ferrous quinolinate is shown here as a 1:1 complex but ferrous ions are also capable of forming 2:1 and 3:1 complexes with chelating compounds. Picolinic and nicotinic acid are isomers which differ from quinolinic acid only in the loss of one of the carboxyl groups.

not affect the inhibition of PEP formation from oxaloacetate by quinolinic acid. The inhibition by quinolinates is not due to any alteration of the pH of the reaction because there was no change in the pH at the beginning or end of the reaction in the presence of these compounds.

A reciprocal plot of the velocity of the reaction against the oxaloacetate concentration in the presence and absence of inhibitor was done in order to further evaluate the mechanism of the inhibition by quinolinates. At a concentration of 1 mM quinolinic acid appears to be a non-competitive inhibitor of PEP carboxylase (Fig. 2). At a higher concentration (2 mM), the inhibition by quinolinic acid is of a mixed type. This may be due to the formation of magnesium quinolinate because the inhibition of PEP carboxylase by manganous quinolinate is mixed (Fig. 3). Ferrous quinolinate is



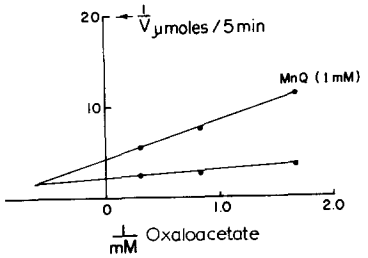
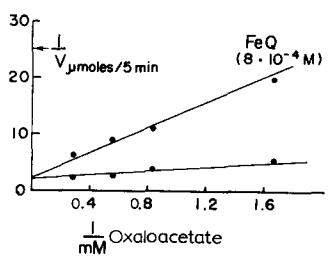




Fig. 2. The assay was performed as listed under Table I in the presence and absence of 1 mM quinolinic acid.

Fig. 3. MnQ is manganous quinolinate.

a more effective inhibitor than the other quinolinates because it is a more truly competitive inhibitor of PEP carboxylase (Figs 4 and 5). Using the data from Figs 2 and 4 an apparent $K_i$ can be calculated for quinolinic acid and ferrous quinolinate (Table III)[8]. The calculated $K_i$ for ferrous quinolinate is 5-fold less than that for quinolinic acid and approx. 4 times the reported $K_m$ of PEP carboxylase for oxaloacetate[9].

The spontaneous decarboxylation of oxaloacetate to pyruvate under these assay conditions occurred at a constant rate of 2.4% · $min^{-1}$ independent of the oxaloacetate concentration or the presence or absence of quinolinic acid. Therefore correcting for



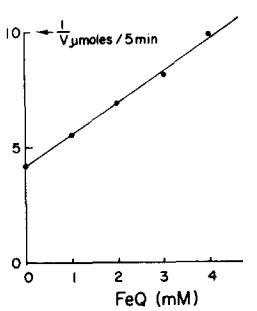




Fig. 4. FeQ is ferrous quinolinate. A ferrous quinolinate concentration of 1 mM also gave a reciprocal plot which intercepted the basal plot on the ordinate.

Fig. 5. Assayed as described in Table I.

this does not change the calculated $K_i$ values derived in Table III or the relative position of the lines in the reciprocal plots. It is of course essential to correct for this in determining the actual $K_m$ for oxaloacetate.

TABLE III

INHIBITION CONSTANTS FOR QUINOLINIC ACID AND FERROUS QUINOLINATE

The $K_i$ for quinolinic acid was calculated from Fig. 2 using the formula for a non-competitive inhibitor ($K_i = I/(V/V_p) - 1$). $V_p$ is the intercept on the ordinate in the presence of the inhibitor, and $I$ is the concentration of the inhibitor. The $K_i$ for ferrous quinolinate was calculated from Fig. 4 using the formula for a competitive inhibitor ($K_i = I/(K_p/K_m) - 1$). $K_p$ is the intercept on the abscissa in the presence of the inhibitor.

| *Compound* | *$K_i$ (calculated) (M)* |
|---|---|
| Quinolinic acid | $5 \cdot 10^{-4}$ |
| Ferrous quinolinate | $1 \cdot 10^{-4}$ |

Manganous chloride will decrease the inhibitory effect of ferrous quinolinate on PEP carboxylase. The degree to which this inhibition is relieved by manganous chloride is also dependent upon the level of oxaloacetate present in the assay system (Table IV). A 4-fold increase in the initial concentration of oxaloacetate made a 3-fold difference in the relief of the ferrous quinolinate inhibition by manganous chloride. (In the absence of ferrous quinolinate the stimulation of PEP carobyxlase by manganous chloride is not dependent upon the level of oxaloacetate)[3].

TABLE IV

THE EFFECT OF OXALOACETATE CONCENTRATION ON THE RELIEF OF FERROUS QUINOLINATE INHIBITION BY MANGANOUS CHLORIDE

The incubation mixture and assay procedure were the same as listed in Table I. The concentration of oxaloacetate was varied and ferrous quinolinate and manganous chloride were added as shown in the Table.

| *Oxaloacetate (mM)* | *Ferrous quinolinate (mM)* | *Manganous chloride* | *Alteration in activity (% change from the basal value)* |
|---|---|---|---|
| 1 | – | – | 0 |
| 1 | 1 | – | −64 |
| 1 | 1 | 1 | −36 |
| 4 | 1 | – | −45 |
| 4 | 1 | 1 | +37 |

DISCUSSION

The data presented demonstrate that the dicarboxylic pyridine derivative quinolinic acid inhibits PEP carboxylase from rat liver cytoplasm by competing with its dicarboxylic substrate oxaloacetate. The divalent manganous and ferrous ions which are chelated between the ring nitrogen and the α-carboxyl group[10] enhance the capacity of quinolinate to competitively inhibit PEP carboxylase by facilitating the attachment of quinolinate to the site on the enzyme for oxaloacetate. (Although the effect

of manganous ions in this regard greatly depends upon the concentration of oxaloacetate.)

Several points indicate that these *in vitro* studies of the effect of quinolinate upon the enzyme at a limiting level of oxaloacetate are pertinent to the effect of tryptophan on gluconeogenesis in the intact animal. The first of these is that the level of oxaloacetate *in vivo* is limiting. (The concentration of oxaloacetate in the liver ranges from $1 \cdot 10^{-6}$–$1 \cdot 10^{-5}$ M[11] whereas the $K_m$ of PEP carboxylase for oxaloacetate is $2.5 \cdot 10^{-5}$ M)[9]. Another point is that a block in the PEP carboxylase step in the perfused liver or the intact animal is associated with a quinolinic acid level in the liver of $1 \cdot 10^{-4}$ M which correlates well with the apparent $K_i$ for ferrous quinolinate that we derived from our kinetic data. The evidence for this statement is this, using a perfused liver Soling *et al.*[12] found that a quinolinate concentration of 1.6 mM in the perfusate produced a 50% fall in the formation of glucose from alanine by the liver of starved rats. This probably represents an intracellular concentration in the range of $1 \cdot 10^{-4}$ M because quinolinic acid does not readily enter the liver. (The gradient between the perfusate and the liver in the studies of Hagino *et al.*[13] was 10 to 1). The intraperitoneal injection of 10 mg of L-tryptophan/100 g of body weight in fasted rats results in a quinolinic acid level in the liver of $43 \pm 8$ $\mu$g/g of liver, wet weight (H. McDaniel, unpublished). Assuming a water content of 70% this represents a quinolinic acid concentration of approx. $3.7 \cdot 10^{-4}$ M. This amount of tryptophan also produces a 5-fold rise in the level of malate indicating that the PEP carboxylase step is blocked (H. McDaniel, unpublished). Lardy[4] has proposed that in addition to direct inhibition of the enzyme quinolinic acid also inhibits PEP carboxylase by chelating ferrous ions and preventing the stimulation that normally occurs at a ferrous ion concentration of $5 \cdot 10^{-5}$ M. If such a mechanism is operative *in vivo* then a quinolinic acid concentration of $1 \cdot 10^{-5}$ M in the liver should also influence this reaction. We have found some increase above the basal content of malate in animals given amounts of tryptophan that produce quinolinic acid levels in this range (H. McDaniel, unpublished). Therefore, this mechanism may also be operative in addition to the direct inhibitory effect of quinolinic acid on the enzyme.

Veneziale *et al.*[14] found that manganous chloride would prevent the inhibitory effect of quinolinic acid on gluconeogenesis *in vivo* and Lardy[4] has recently reported that manganous ions reverse the inhibition of PEP carboxylase by ferrous quinolinate *in vitro*. He has suggested that this is due to the displacement of the ferrous ions by manganous ions forming manganous quinolinate which is not inhibitory. There is a greater drop in the pH with the addition of ferrous chloride to a solution of quinolinic acid than on the addition of manganous chloride (H. McDaniel, unpublished) indicating that the ferrous ion forms a more stable complex with quinolinic acid[15]. Thus it is unlikely that manganous ions are displacing the ferrous ions and even if they did manganous quinolinate is also inhibitory at limiting concentrations of oxaloacetate. The manganous ions may be acting by shifting the balance in favor of oxaloacetate in its competition with ferrous quinolinate for attachment on the active site of the enzyme. The increase in carboxylase activity which Foster *et al.*[16] found in tryptophan treated animals returned to control levels with storage unless dithiothreitol was included in the storage buffer. Lardy[4] has subsequently reported activation of the enzyme by ferrous quinolinate *in vitro* and proposed that this was due to tranfer of ferrous ions from the quinolinate to the enzyme on dilution. We have also obtained activa-

tion of PEP carboxylase *in vitro* by reacting the enzyme with sodium borohydride (H. McDaniel, unpublished). The sodium borohydride activated enzyme resembles the quinolinate activated enzyme in that it is not further stimulated by manganese. We would propose that the activation by ferrous quinolinate and sodium borohydride is due to the reduction of a disulfide group at the active site of the enzyme which then participates in the reaction without requiring a metal ion (manganous or ferrous). Further data is needed to define what the actual activation process is with ferrous quinolinate and sodium borohydride.

Several factors will alter the degree of inhibition of gluconeogenesis by quinolinic acid. In those animals with PEP carboxylase located primarily in the mitochondria quinolinic acid is much less effective. As shown by Soling *et al.*[12] this is probably due to the failure of quinolinic acid to readily enter the mitochondria and inhibit the mitochondrial PEP carboxylase. This same line of reasoning would account for the further decrease in the output of glucose by the perfused liver on the addition of fatty acids or amino acids[14] since both of these substrates would tend to decrease the intramitochondrial concentration of oxaloacetate through an increase in the conversion of oxaloacetate to malate and aspartate[11]. Therefore less oxaloacetate would be available for conversion to PEP by the mitochondrial PEP carboxylase. The inhibitory effect of large amounts of L-tryptophan on gluconeogenesis may have components other than inhibition of PEP carboxylase by quinolinate since ATP[17] and cyclic AMP[18] levels fall following the injection of large amounts of L-tryptophan to rats.

On examining quinolinic acid levels in the liver during starvation and re-feeding we have found a concentration in the range of $1 \cdot 10^{-5}$ M with re-feeding (H. McDaniel, unpublished). A quinolinic acid level of $1 \cdot 10^{-4}$ M was found only after the intraperitoneal injection of 10 mg or more of L-tryptophan/100 g of body weight (H. McDaniel, unpublished). If quinolinic acid does prevent the normal stimulation of PEP carboxylase activity by ferrous ions as Lardy[4] has proposed then a concentration of $1 \cdot 10^{-5}$ M would have a great deal of significance in regulating the activity of the cytoplasmic PEP carboxylase. The importance of this is readily apparent on examining the effect of diabetes mellitus and chronic hydrocortisone administration on the formation of quinolinic acid from tryptophan. Both of these abnormal states are associated with a marked increased in the overall gluconeogenic capacity of the liver and a marked decrease in the formation of quinolinic acid from a given dose of tryptophan when compared to the normal rat (H. McDaniel, unpublished). If this mechanism is not operative and quinolinic acid influences PEP carboxylase only by direct inhibition then the effect of this compound on gluconeogenesis is a pharmacological one and would only be seen under unusual circumstances.

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