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Structural Requirements for Pyridine Carboxylate Effects on Phosphoenolpyruvate Carboxykinase[1,2]

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It has previously been established that quinolinic acid and 3-mercaptopicolinic acid cause hypoglycemia by inhibiting Phosphoenolpyruvate (PEP) carboxykinase and gluconeogenesis. In the rat, 3-aminopicolinic acid permits Fe^{2+} to activate this enzyme; it enhances gluconeogenesis and causes hyperglycemia. In the present study, other pyridine carboxylates were screened for effects on the activity of PEP carboxykinase. The structural requirement for an inhibitor or an activator of this enzyme has been defined: It must be a picolinic acid derivative with the α carboxyl unsubstituted and with another group on position 3. The group at position 3 determines the effect (inhibition or activation) and the potency of the compound. Compounds such as picolinic acid, all the isomers of quinolinic acid, 2-mercaptonicotinic acid, and 2-aminonicotinic acid were inactive. Fe^{2+} enhances the potency of quinolinate and 2-mercaptopicolinate 15- to 20-fold, and 3-aminopicolinate does not activate the carboxykinase in the absence of Fe^{2+}. It is therefore assumed that Fe^{2+} binds to the ring nitrogen and the α-carboxyl group of one or more molecules of these compounds to form an effective coordination complex. Complexes involving picolinate derivatives with an acidic function at position 3 inhibit; the complex of Fe^{2+} with 3-aminopicolinate either delivers Fe^{2+} to the catalytic site and then dissociates or the Fe^{2+} in the complex is catalytically active. 3-Aminopicolinate causes hyperglycemia in the guinea pig. It activates guinea pig liver cytosolic PEP carboxykinase *in vitro* but does not activate the mitochondrial carboxykinase. If activation of PEP carboxykinase is the means by which 3-aminopicolinate causes hyperglycemia, our findings indicate that the cytosolic enzyme can play an important role in glucose synthesis in species having appreciable amounts of both carboxykinases.

INTRODUCTION

Studies on the influence of tryptophan administration on the cytosolic phosphoenolpyruvate (PEP) carboxykinase (*1–4*) of rat liver led to the finding that this enzyme is activated by Fe^{2+} (*5*) but only in the presence of a specific protein, PEP carboxykinase ferroactivator (*6*, *7*). This enzyme is inhibited by quinolinic acid and 3-mercaptopicolinic acid (*4*, *5*, *8–10*). When administered to rats, these compounds cause hypoglycemia (*11*). The closely related compound, 3-aminopicolinic acid, causes hyperglycemia in the rat (*12*, *13*) by enhancing gluconeogenesis (*13*). 3-Aminopicolinate was found to activate PEP carboxykinase in the presence of Fe^{2+} and appears to be a model for studies of the mode of action of the ferroactivator protein (*14*).

[1] We are grateful for Professor W. S. Johnson's contributions to chemistry at Wisconsin in an early phase of his career and are pleased to pay tribute to him on the occasion of his 65th birthday.

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The effects of these three pyridine derivatives reflect structural specificity and are not merely the result of metal chelation. Pyridine dicarboxylic acids other than quinolinate do not inhibit PEP carboxykinase activity significantly, and a variety of specific iron chelators were also without effect (*5*). The ability of 3-aminopicolinate to mimic the effect of ferroactivator protein on PEP carboxykinase is a further indication that certain pyridine carboxylates probably have specific affinity for the site on the enzyme that binds divalent transition metals.

In this paper we report the results of experiments designed to examine further the relation between the structure of compounds and their effects on the activity of PEP carboxykinase. The effects of 3-aminopicolinate on the blood glucose and on its specificity toward the cytosolic and mitochondrial PEP carboxykinases of the guinea pig are also reported.

EXPERIMENTAL PROCEDURE

Materials. Materials were from the following sources: Na_3ITP, Na_2ADP, NADH, and dithiothreitol, P–L Biochemicals Inc. (Milwaukee, Wisconsin); oxalacetate, Calbiochem (San Diego, California); Hepes[3] and TEA,[3] Sigma Chemical Co. (St. Louis, Missouri), ammonium sulfate (enzyme grade) and Tris,[3] Schwarz/Mann; glucose oxidase for the determination of blood glucose (*15*), lactate dehydrogenase, and pyruvate kinase, Boehringer–Mannheim (Indinapolis, Indiana); DEAE[3]–cellulose (DE 32), Whatman Co., Na EDTA,[3] Baker Chemical Co; quinolinic acid, K & K Laboratories (Plainview, New York). 3-Mercaptopicolinic acid and 3-aminopicolinic acid hydrochloride were the generous gifts of Dr. Harry Saunders and T. Resnick of Smith Kline Corp. (Philadelphia, Pennsylvania). All sulfano pyridine carboxylic acids were the generous gifts of Dr. J. Delarge and Prof. C. L. Lapiére, University of Liege, Belgium. Quinolinic acid dihydrazide was a generous gift of Dr. A. Gold, Syracuse Cancer Research Institute. 2-Mercaptonicotinic acid was synthesized from NaSH and 2-chloronicotinic acid (*16*). Methyl oxalahydroxamic acid was synthesized from methyloxalyl chloride and hydroxylamine hydrochloride (*17*). All other compounds were of the highest purity available from Aldrich Chemical Co. (Milwaukee, Wisconsin) or Sigma Chemical Co. and were used as received.

Enzyme preparations. Rat liver cytosolic PEP carboxykinase was purified by the method of Ballard and Hanson (*18*, *19*) with some modifications (*20*). The specific activities of the enzyme preparations used in this study were 4.8, 6.2, and 6.2 μmol $\times$ min^{-1} $\times$ milligrams $protein^{-1}$ when assayed in the direction of PEP formation at 25°C without prior incubation with effectors. Rat liver cytosol was the supernatant fraction obtained from centrifuging a 1:3 (w/v) homogenate of liver from 24-hr starved rats in 0.25 *M* sucrose, 10 m*M* TEA–Cl (pH 7.5) at 105 000 *g* for 1 hr.

Guinea pig liver cytosolic PEP carboxykinase was partially purified to remove ferroactivator protein in the following way: Five male 550-g guinea pigs were starved for 72 hr and then killed by decapitation. All subsequent steps were carried out at 4°C. The livers were perfused *in situ* with 0.9% NaCl to remove blood, excised, and homo-

[3] Abbreviations used: EDTA, ethylene diaminetetraacetate; DEAE, diethylaminoethyl; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TEA, triethanolamine; and Tris, Tris(hydroxymethyl)-aminomethane.

genized in 3 vol (w/v) of 10 m*M* TEA–Cl (pH 7.5), 0.25 *M* sucrose, and 2.5 m*M* EDTA. The part of the liver supernatant fraction (from centrifuging at 105 000 *g* for 1 hr) that was precipitated by 40 to 70% saturation with ammonium sulfate was dialyzed overnight vs two changes of 50 vol of 20 m*M* Tris–Cl (pH 7.0), 1 m*M* dithiothreitol, and 1 m*M* EDTA and applied to a DEAE–cellulose column (2.5 cm^2 × 16 cm). Ferroactivator protein did not adhere to the column. Phosphoenolpyruvate carboxykinase was eluted from the column with a 0 to 0.2 *M* KCl gradient in 20 m*M* Tris–Cl (pH 7.0, 0.1 m*M* dithiothreitol, and 0.1 m*M* EDTA. The enzyme eluted before 0.1 *M* KCl.

Mitochondria were isolated from the original homogenate of guinea pig liver described above according to the method of Johnson and Lardy (*21*) except that the mitochondrial pellet was washed four additional times. Mitochondria were lysed by freeze–thawing three times in 5 m*M* Hepes (pH 7.5). The supernatant fraction obtained from centrifuging the lysate at 30 000 *g* for 15 min was used for assays in which mitochondrial PEP carboxykinase was required.

Phosphoenolpyruvate carboxykinase activities. Enzyme activity was assayed in the direction of PEP formation in two ways. All rates, except those depicted in Figs. 3 and 4, were determined by the method of Seubert and Huth (*22*) in a final volume of 1.0 ml of 1.5 m*M* oxalacetate, 2 m*M* ITP, 3 m*M* $MgCl_2$, and 50 m*M* Hepes–NaOH (pH 7.5) at 25°C. The reaction was initiated by addition of enzyme and terminated by adding 10 mg of KBH_4. A standard reaction time of 5 min was used. The reaction mixture was deproteinized with $HClO_4$ and neutralized with KOH. Phosphoenolpyruvate generated by the reaction was determined by a standard enzymatic assay (*23*). Enzyme rates were linear under all conditions described in this report.

The rates shown in Figs. 3 and 4 were determined with a continuous spectrophotometric assay at 340 nm. The reaction mixture contained, in a final volume of 1.0 ml, 50 μM oxalacetate (when its concentration was not varied), 2 m*M* ITP, 1.8 m*M* ADP, 4.4 m*M* $MgCl_2$, 0.14 m*M* NADH, 50 m*M* Hepes–KOH (pH 7.5), 5.5 U of lactate dehydrogenase (free of malic dehydrogenase) and 4 U of pyruvate kinase. The assay was performed at 25°C. The rate of NADH disappearance in the absence of PEP carboxykinase was subtracted from the total rate to obtain the rate attributable to enzyme catalysis.

Preliminary incubation of enzyme with metals. Prior to enzymatic assay, metal salts and/or organic compounds were incubated with 2.4 to 3.5 μg of purified rat liver PEP carboxykinase or 10 to 30 μl of rat liver cytosol or guinea pig liver fractions at 0°C for 10 min in a final volume of 0.2 ml containing 0.8 m*M* dithiothreitol and 4.2 m*M* Hepes–NaOH (pH 7.5) as previously described (*6, 20*). Incubations were terminated by adding 0.1 ml of the incubation mixture to 0.9 ml of enzyme reaction mixture in both assays used.

Treatment of data. For plots involving reciprocal velocities, straight lines were fit to the points by linear regression analysis, weighting each point by a factor containing V^4. Curved lines were drawn to fit points when plots were not linear.

RESULTS

Screening of compounds. The specific structural requirements for pyridine derivatives to have an effect on PEP carboxykinase activity can be deduced from Tables

TABLE 1

COMPOUNDS TESTED FOR AN ABILITY TO INHIBIT PHOSPHOENOLPYRUVATE CARBOXYKINASE[a]

Compound[b]	K_i	
	No Fe^{2+}	With Fe^{2+}
Quinolinic acid	1.5 m*M*	0.1 m*M*
3-Mercaptopicolinic acid	0.1 m*M*	7 μM
2-Mercaptonicotonic acid	N	N
3-Sulfanopicolinic acid	2.0 m*M*	0.4 m*M*
2-Sulfanonicotinic acid	N	N
Quinolinic acid dihydrazide	0.5 m*M*	0.5 m*M*
2,3-Pyrazine dicarboxylic acid	1.0 m*M*	0.6 m*M*
3-Hydroxypicolinamide	N	0.7 m*M*
3-Hydroxypicolinic acid	N	1.5 m*M*
Quinolinic acid α-methyl ester	N	N

[a] Compounds added to the enzyme assay mixture were tested on rat liver cytosol that was incubated with and without 30 μM $FeCl_2$ prior to assaying for enzyme activity. The concentration of inhibitors required to give 50% inhibition (K_i value) was determined by a Dixon plot. The letter "N" indicates no effect. Maximum concentrations tested were from 1 to 10 m*M*. The numbers in parentheses refer to previously published work on compounds that were retested.

[b] Other compounds ineffective with or without Fe^{2+}: picolinic acid (*5*), pyridine 2,4-dicarboxylic acid (*5*), pyridine 2,5-dicarboxylic acid (*5*), pyridine 2,6-dicarboxylic acid (*5*), anthranilic acid (*5*), 3-hydroxyanthranilic acid (*5*), quinolinic acid dimethyl ester, quinolinic acid α-ethyl ester, methyliminopicolinic acid, 2-mercaptopyridinol, 2-pyridine aldoxime, 2-mercaptopyridine, 2,3-disulfanopyridine, 3-sulfano 2-chloropyridine, 2-chloronicotinic acid, fusaric acid (5-butyl picolinic acid), pyridoxal phosphate, oxamic acid hydrazide, oxalyl dihydrazide, acethydrazide, methyloxalohydroxamic acid, α-mercaptobenzoic acid, salicylhydroxamic acid, *p*-aminonicotinamide, 2-piperazine 3H,4-quinalazone, 2-thiophenecarboxylic acid, 2-thiophenecarboxylic acid hydrazide, 6-amino-5-nitrothiouracil, 3-indolacetic acid, ethylindol 2-carboxylic acid, 5-nitrocytosine, 5-aminoorotic acid, the ionophore A23187, ferrocene, 1,1-ferrocenedicarboxylic acid, oxytetracycline, and chlortetracycline.

1 and 2. For significant inhibition, a carboxyl group must be on position 2 and a second acidic group must be on position 3 as in quinolinic acid, 3-mercaptopicolinic acid, and 3-sulfanopicolinic acid (Table 1). 3-Aminopicolinic acid with its carboxyl group on position 2 and a potentially basic group on position 3 provides the only example of an activating molecule. Transposing the groups on carbons 2 and 3 of these compounds as in the nicotinate derivatives 2-mercaptonicotinic acid, 2-sulfanonicotinic acid (Table 1), and 2-aminonicotinic acid (Table 2) results in complete loss of the inhibiting or activating properties. The lack of activity of the α-methyl and α-ethyl esters of quinolinic acid (Table 1) also shows the importance of the free carboxyl group at position 2. The importance of the group on position 3 is demonstrated by the fact that neither picolinic

TABLE 2

COMPOUNDS TESTED FOR AN ABILITY TO PERMIT Fe^{2+} TO ACTIVATE PHOSPHOENOLPYRUVATE CARBOXYKINASE[a]

Compound[b]	Activity (% of control)[c]
3-Aminopicolinic acid	271[d]
2-Aminonicotinic acid	Does not activate
3-Hydroxypicolinamide	132
5-Butylpicolinic acid	144
2,3-Disulfanopyridine	122

[a] Pyridine derivatives (1 m*M*) were incubated with PEP carboxykinase purified from rat liver cytosol in the standard incubation mixture with 200 μM $FeCl_2$ for 10 min prior to assaying for enzyme activity. The incubation mixture was diluted 1 : 10 upon addition to the enzyme assay mixture.

[b] Compounds (0.1 to 1.0 m*M*) not permitting any activation by 30 to 200 μM Fe^{2+}: salicylhydroxamic acid, 6-aminonicotinamide, 3-aminophthalic acid, 1,1′-ferrocenedicarboxylic acid, ferrocene, 4-hydroxyphthalic acid, *p*-aminosalicyclic acid, 5-nitrocytosine, 5-aminoorotic acid, A23187, 3-aminophthalhydrazide, 3-sulfanopicolinic acid, 2-sulfanonicotinic acid, 3-sulfano 2-chlorpyridine, anthranilic acid, hydroxyanthranilic acid, quinolinic acid dihydrazide, picolinic acid, nicotinic acid, 2-pyrazine dicarboxylic acid, 2,4-pyridine dicarboxylic acid, 2,5-pyridine dicarboxylic acid, 2,6-pyridine dicarboxylic acid, 3-hydroxypicolinic acid, 6-amino-5-nitrothiouracil, 3-indolacetic acid, ethylindol 2-carboxylic acid, 8-hydroxyquinoline, and 8-hydroxyquinaldine.

[c] Activity of the control enzyme incubated with 200 μM Fe^{2+} is designated 100%. Lesser activation was obtained with 0.1 m*M* concentrations of the pyridine derivatives and 30 μM Fe^{2+}.

[d] The concentration required for one-half maximal activation was 20 to 50 μM.

acid nor any of its derivatives with a carboxyl group at positions 4, 5, or 6 has significant activating or inhibiting properties (Tables 1 and 2). In agreement with the above, Blank *et al.* (*12*) have shown that none of nine isomers of 3-mercaptopicolinate, including 2-mercaptonicotinate, is a hypoglycemic agent.

The fact that α-mercaptobenzoic acid and anthranilic acid, compounds that have sulfhydryl and amino groups adjacent to a carboxyl group on a benzene ring, do not inhibit (Table 1) or activate (Table 2) the enzyme indicates that the ring nitrogen of the two former compounds is necessary for activity. Numerous other compounds, many of them chelating agents and structurally resembling the active pyridine carboxylates, had no activity (Tables 1 and 2). Some of these compounds such as 2-thiophenecarboxylic acid (*24*), 2-piperazino-3H,4-quinazolone (*25*), and oxytetracycline (*26*) are known to be hypoglycemic agents, and others such as 6-aminonicotinamide (*27*), 8-hydroxyquinoline (*28*), and 3-indolacetic acid (*29*) are known to be hyperglycemic agents. Fusaric acid (5-butylpicolinic acid) is a potent inhibitor of dopamine hydroxylase (*30*), a copper-containing enzyme.

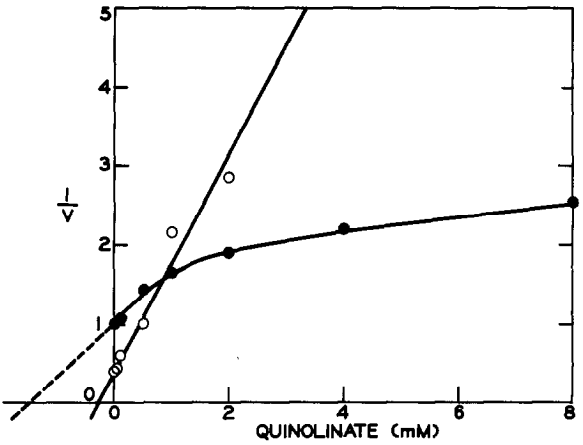

FIG. 1. Effect of Fe^{2+} on inhibition of PEP carboxykinase by quinolinic acid. Rat liver cytosol was incubated without (●) and with (○) 30 μM $FeCl_2$ in the standard incubation mixture prior to assaying for enzyme activity. The three lowest reciprocal velocity values were used to fit a straight line to the curve for cytosol not incubated with Fe^{2+} (dashed line). The concentration of quinolinate in the enzyme assay mixture is shown on the abscissa.

Effect of Fe^{2+} on potency of compounds. Previous work has shown that incubation of crude PEP carboxykinase with Fe^{2+} prior to assaying for activity increased its activity three- to fourfold and also potentiated inhibition by quinolinic acid (*5*). Figure 1 is a Dixon plot that shows how incubating crude rat liver cytosol with 30 μM Fe^{2+} prior to assaying for enzyme activity increases the inhibitory potency of quinolinate 15 times (K_I without Fe^{2+} is 1.5 mM and with Fe^{2+} it is 0.1 mM). Moreover, inhibition of the enzyme not activated by Fe^{2+} is incomplete as previously described (*5*), but in contrast to an early finding (*5*) we now find that inhibition of the enzyme activated by Fe^{2+} is complete, provided a sufficiently high concentration (2–4 mM) of quinolinate is used.

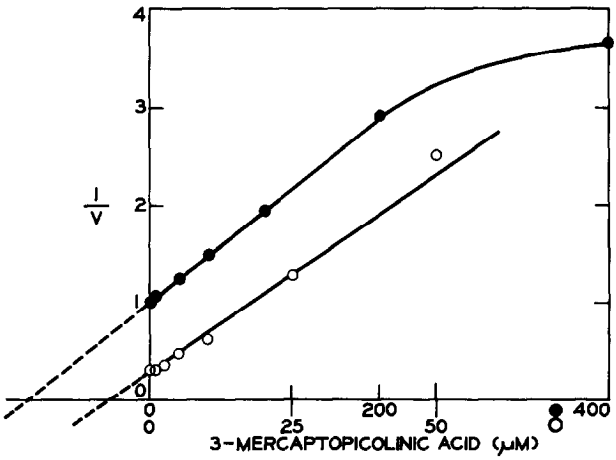

FIG. 2. Effect of Fe^{2+} on inhibition of PEP carboxykinase by 3-mercaptopicolinic acid. Conditions were the same as those given in the legend to Fig. 1: without (●) and with (○) Fe^{2+}. The concentration of 3-mercaptopicolinate in the enzyme assay mixture is shown on the abscissa.

Similar results were obtained with 3-mercaptopicolinate; the K_i's were 100 and 5 μM before and after incubating cytosol with Fe^{2+}, and inhibition in the absence of Fe^{2+} was only partial (Fig. 2).

When PEP carboxykinase is purified, ferroactivator protein is removed and the enzyme is no longer activated by Fe^{2+} (*6*). Sulfate ion can permit maximal (but

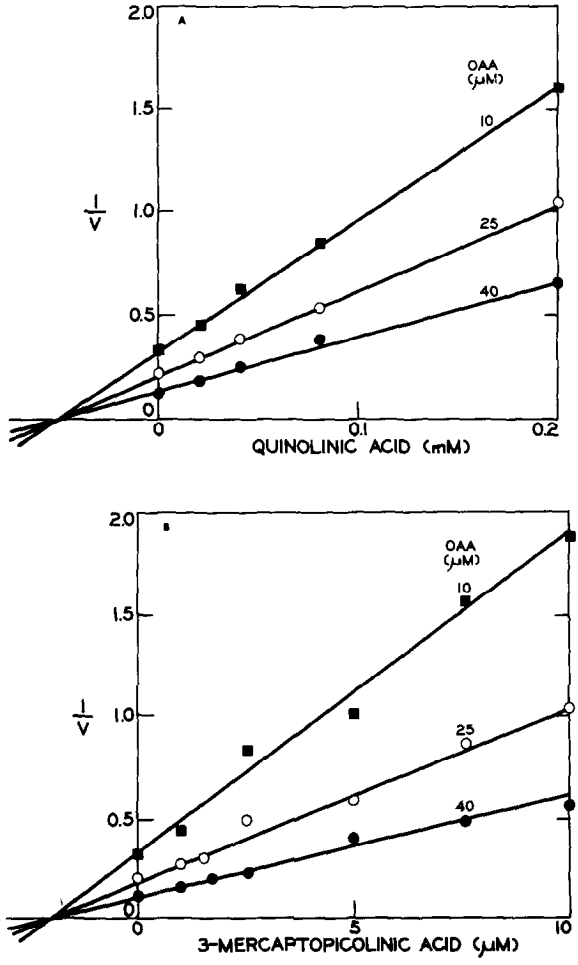

FIG. 3. Determination of K_i's of quinolinic acid and 3-mercaptopicolinic acid for Fe^{2+}-activated PEP carboxykinase. Purified rat liver PEP carboxykinase was incubated with 30 μM Fe^{2+} and 1 mM Na_2SO_4 in the standard incubation mixture before assaying for enzyme activity in the presence of various concentrations of oxalacetate. The concentrations of quinolinate (A) and 3-mercaptopicolinate (B) in the enzyme assay mixture are shown on the abscissa.

transient) activation of the enzyme by Fe^{2+} (*20*). The inhibition constants of quinolinate and 3-mercaptopicolinate for purified rat liver cytosol PEP carboxykinase were determined by preactivating the enzyme with 30 μM Fe^{2+} and 1 mM inorganic sulfate and varying the concentration of oxalacetate in the enzyme reaction mixture. The apparent K_i's for quinolinate and 3-mercaptopicolinate were 50 and 2 μM, respectively

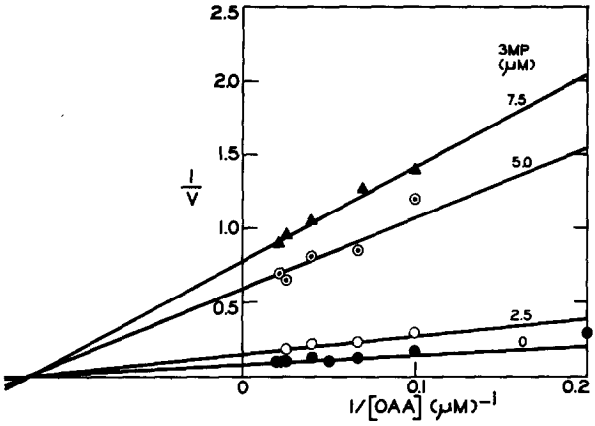

FIG. 4. Lineweaver–Burk plot of the effect of 3-mercaptopicolinic acid (MP) on the initial velocities of Fe^{2+}-activated PEP carboxykinase assayed with various oxalacetate (OAA) concentrations. Conditions were the same as those given in legend to Fig. 3.

(Fig. 3). If the enzyme was not preactivated by Fe^{2+}, the K_i's for quinolinate and 2-mercaptopicolinate were about 1.0 m*M* and 45 μM, respectively (data not shown).

McDaniel *et al.* (*8*) have reported that adding Fe^{2+} to the enzyme reaction mixture converts the inhibition pattern obtained with quinolinate from noncompetitive to one competitive with respect to oxalacetate. We have not been able to observe this with quinolinate, 3-mercaptopicolinate, or pyridine 2,3-dihydrazide if Fe^{2+} has been added to the enzyme reaction mixture or incubated with the enzyme before assaying for activity. Figure 4 shows conclusively that 3-mercaptopicolinate is a noncompetitive inhibitor of the Fe^{2+}-activated enzyme with respect to oxalacetate.

To determine whether any of the inhibitors acted irreversibly, the enzyme was incubated with 30 μM Fe^{2+} and 1 m*M* quinolinate, 3-mercaptopicolinate, or pyridine 2,3-dihydrazide for periods of up to 1 hr. The inhibition exerted by these compounds was completely reversed by dialyzing the enzyme for 24 hr (data not shown). Incubating the enzyme with 3-mercaptopicolinate in the presence or absence of Fe^{2+} for increasing periods of from 1 to 15 min did not produce increasing inhibition of the enzyme (data not shown).

Effect of 3-aminopicolinate on the blood glucose and PEP carboxykinase of the guinea pig. Evidence has previously been presented that indicates that 3-aminopicolinate causes hyperglycemia in the rat by permitting a metal ion to activate cytosolic PEP carboxykinase (*13*). Guinea pig liver contains a very active mitochondrial PEP carboxykinase as well as the distinct cytosolic enzyme. Figure 5 shows that 3-aminopicolinate causes hyperglycemia in the guinea pig. To determine whether either or both of the guinea pig hepatic PEP carboxykinases are involved in producing the hyperglycemia, the response of both enzymes to 3-aminopicolinate was examined. Guinea pig cytosolic PEP carboxykinase was partially purified to remove ferroactivator protein, which permits Fe^{2+} to activate the enzyme (*6*, *7*), in order to determine whether 3-aminopicolinate could permit Fe^{2+} to activate the enzyme. The mitochondrial enzyme did not require purification, for mitochondria contain only small amounts of ferroactivator (*31*). Figure 6 shows that 3-aminopicolinate permits Fe^{2+} to

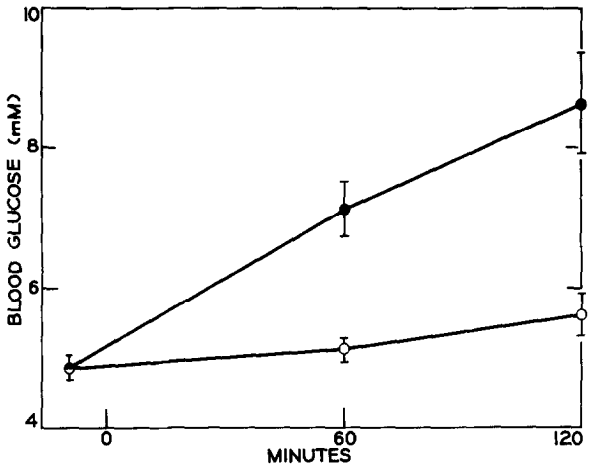

FIG. 5. Effect of 3-aminopicolinate on the blood glucose level of guinea pigs. 3-Aminopicolinate (pH 7.4) (40 mg in 1 ml of H_2O) or 0.9% NaCl (1 ml) was injected into the intraperitoneal cavity of alert unanesthetized 48-hr-starved 200- to 250-g guinea pigs. Blood samples (50 μl) obtained before and at intervals after the injections were immediately placed into 0.95 ml of cold 6% perchloric acid for glucose determination. The results given are the mean ± SEM from three animals treated with 3-aminopicolinate (●) and four animals treated with NaCl (○).

enhance the initial velocity of partially purified guinea pig cytosolic PEP carboxykinase three- to fourfold. In fact, the curves in Fig. 6 are almost superimposable upon those obtained with purified rat liver cytosolic PEP carboxykinase under identical experimental conditions (*14*). The slight activation seen in Fig. 6 with high concentrations of 3-aminopicolinate in the absence of added Fe^{2+} could have been due

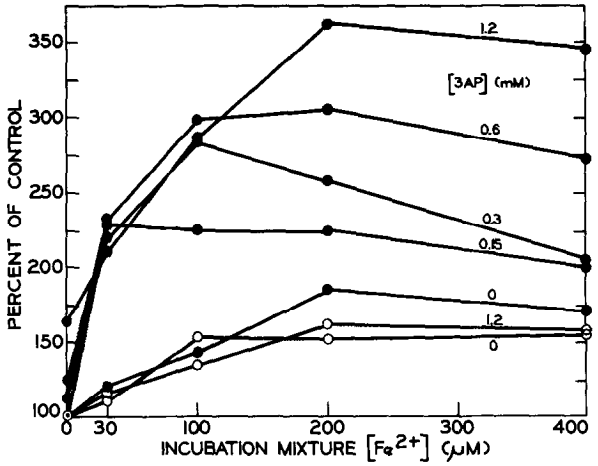

FIG. 6. Effect of 3-aminopicolinate upon the initial velocities of guinea pig liver cytosolic and mitochondrial PEP carboxykinases. Partially purified cytosolic PEP carboxykinase (●) or mitochondrial fraction (○) was incubated with various concentrations of 3-aminopicolinate and/or $FeCl_2$ in the standard incubation mixture for 10 min prior to assaying for enzyme activity. Adding incubation mixture to the enzyme reaction mixture resulted in a 1 : 10 dilution of the incubation mixture. The concentration of 3-aminopicolinate (3AP) is shown in parentheses near each curve. Each point represents the mean of two to six observations.

to metal ions contaminating the partially pure preparation. This is not seen with the purified rat liver enzyme (*14*). 3-Aminopicolinate does not permit Fe^{2+} to activate the mitochondrial enzyme (Fig. 6).

DISCUSSION

The results of this study show that for a compound to influence the activity of rat liver cytosolic PEP carboxykinase, it must be a picolinic acid derivative with a substituent at position 3. To date, the compounds found to have the most influence on the activity of the enzyme are quinolinic acid, 3-mercaptopicolinic acid, and 3-aminopicolinic acid (*5*, *7–11*, *14*) (Tables 1 and 2). The first two compounds inhibit the enzyme (*7*, *9–11*) (Figs. 1–4) and gluconeogenesis (*1–4*, *9–11*, *32*, *33*) and cause hypoglycemia (*11*). 3-Aminopicolinic acid permits metal ions to activate the cytosolic enzyme from rat liver (*14*) and from guinea pig liver (Fig. 6), enhances gluconeogenesis (*13*), and causes hyperglycemia (*13*) (Fig. 5).

The previously mentioned configuration is essential for activity, probably because it can permit the coordination of a metal ion between the ring nitrogen and the α-carboxyl group. This type of bonding between picolinate derivatives and transition metals is common (*34*, *35*). The substituent on carbon 3 determines the effect (inhibition or activation) the compound will have on PEP carboxykinase and its potency. Quinolinate (*5*, *8*) and 3-mercaptopicolinate are more potent inhibitors of PEP carboxykinase when it has been activated by Fe^{2+} prior to assaying for activity (Figs. 1 and 2) or by adding Fe^{2+} to the enzyme reaction mixture (*8* and unpublished data). That activation itself does not in some way improve the sensitivity of the enzyme to these compounds is supported by experiments showing that quinolinate (*5*, *7*, *14*) and 3-mercaptopicolinate (*14*) are less potent inhibitors when the enzyme has been activated by Mn^{2+}. Mn^{2+} also permits maximal activation of the enzyme (*5*, *7*, *14*). Mercaptopicolinate is still a potent inhibitor of the enzyme when its concentration is lower than that of the Fe^{2+} present (*14*) (Figs. 3B and 4). These facts and the fact that quinolinate and 3-mercaptopicolinate are no more potent as metal chelating agents (*14*) than is picolinic acid or any of the isomers of quinolinic acid (*36*), all of which do not inhibit PEP carboxykinase, indicate that their mechanism of inhibition does not involve merely removing an enzyme-bound metal. That Fe^{2+} increases the affinity of quinolinate and 3-mercaptopicolinate (Figs. 1 and 2) for PEP carboxykinase some 15- to 20-fold indicates than an Fe^{2+}-pyridine carboxylate complex is formed and that it is this complex that inhibits the enzyme.

3-Aminopicolinate has no effect on PEP carboxykinase in the absence of a divalent transition metal ion, which demonstrates that it does not activate the enzyme by the withdrawal of an inhibitory metal (*14*) (Fig. 6). These results indicate that a pyridine carboxylate–metal ion complex is formed in a manner analogous to that of the inhibitors, but in this case the complex activates the enzyme. Whether the enzyme is activated directly by binding an Fe^{2+}–3-aminopicolinate complex or by Fe^{2+} transferred to the enzyme from such a complex has yet to be determined. We favor the theory that an Fe^{2+} complex is bound to the enzyme for if Fe^{2+} were transferred to the enzyme from such a complex, 3-aminopicolinate should act catalytically and we have seen no evidence for this. If 3-aminopicolinate were merely improving the stability of Fe^{2+} in solution (Fe^{2+} is readily oxidized to Fe^{3+} which rapidly forms the insoluble polymers at a

neutral pH), most of the pyridine carboxylate derivatives and other moderately strong chelating agents listed in Tables 1 and 2 should permit Fe^{2+} to activate PEP carboxykinase.

Pyridine carboxylate inhibitors are thought to act primarily on cytosolic PEP carboxykinase in the perfused liver (*37*) and in enzyme assays (*38*) and to have relatively little effect on the mitochondrial enzyme. The present work shows that 3-aminopicolinate permits Fe^{2+} to activate only the cytosolic carboxykinase in guinea pig liver (Fig. 6). This was not unexpected since it was previously shown that the cytosolic enzyme but not the mitochondrial enzyme in livers of five mammalian and two avian species was activated by Fe^{2+} and ferroactivator protein (*31*). The effects of the pyridine carboxylate derivatives on the blood glucose level of intact animals (*11*, *13*) and on gluconeogenesis rates (*4*, *10*, *13*) correlate with the effects of the derivatives on cytosolic PEP carboxykinase. This supports other evidence[4] that the cytosolic carboxykinase is more directly involved with adaptive responses in glucose synthesis than is the intramitochondrial enzyme.

In summary, the data indicate that certain Fe^{2+}–pyridine carboxylate complexes influence PEP carboxykinase activity both *in vitro* and *in vivo*. Whether the differences in actions between quinolinate, 3-mercaptopicolinate, and 3-aminopicolinate are due to different configurations of their Fe^{2+} complexes or whether the groups on position 3 cause different interactions of the enzyme with structurally very similar complexes is under investigation.

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