Hyperglycaemic Activity and Metabolic Effects of 3-Aminopicolinic Acid

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Administering 3-aminopicolinate to rats starved for 24 h immediately initiated a progressive increase in blood glucose concentration. Hyperglycaemia was not the result of glycogenolysis, nor was it due to an inhibition of insulin release, since it caused marked hyperinsulinaemia. The rate of [6-³H]glucose disappearance from the blood of the intact rat was not altered by 3-aminopicolinate, indicating that it does not cause hyperglycaemia by inhibiting glucose utilization or by causing a redistribution of total body glucose. 3-Aminopicolinate increased the rate of fall in the specific radioactivity of blood [6-³H]-glucose, indicating dilution of the glucose pool by newly synthesized glucose. The rate of ¹⁴C incorporation into blood glucose from [¹⁴C]alanine and [¹⁴C]lactate was increased 90 and 35% respectively, whereas that from [¹⁴C]glycerol and [¹⁴C]xylitol was either unaffected or slightly decreased by 3-aminopicolinate administration. Liver phosphoenolpyruvate of rats was increased to four to seven times the normal concentration 10 min to 1 h after injections of 50–300 mg of 3-aminopicolinate/kg body wt. and the amounts of 2-phosphoglycerate and 3-phosphoglycerate were increased to three to four times normal. The high concentrations of liver phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate, as well as the enhancement of gluconeogenesis from lactate and alanine, but not from glycerol or xylitol, is compatible with an enhancement of gluconeogenesis at a step between pyruvate and the triose phosphates. After injections of 3-aminopicolinate, liver malate, citrate, aspartate, alanine, lactate and pyruvate were also increased, but to lesser extents than was phosphoenolpyruvate. The increases in some of these metabolites were approximated after an intravenous infusion of glucose, so their elevated concentration after 3-aminopicolinate administration could have been, in part, a consequence of the hyperglycaemia. The possibility is considered that 3-aminopicolinate stimulates gluconeogenesis *in vivo* by facilitating Fe²⁺ activation of phosphoenolpyruvate carboxykinase as it does with the purified enzyme *in vitro* [MacDonald & Lardy (1978) *J. Biol. Chem.* **253**, 2300–2307]. In this effect 3-aminopicolinate may simulate the physiological role of the naturally occurring ferroactivator protein [Bentle & Lardy (1977) *J. Biol. Chem.* **252**, 1431–1440].

The discovery that administration of tryptophan to rats inhibited gluconeogenesis at the site of phosphoenolpyruvate carboxykinase (EC 4.1.1.32) (Foster *et al.*, 1966; Ray *et al.*, 1966) led to the finding that it is quinolinate produced from tryptophan that blocks gluconeogenesis (Veneziale *et al.*, 1967). Further investigation disclosed that Fe²⁺ and Mn²⁺ activate phosphoenolpyruvate carboxykinase and that quinolinate inhibits this enzyme only in the presence of Fe²⁺ (Snoko *et al.*, 1971; Bentle & Lardy, 1977). The fact that quinolinate inhibits the enzyme *in vivo* and that Mn²⁺ reverses quinolinate inhibition of gluconeogenesis in perfused liver (Veneziale *et al.*, 1967) led us to conclude that Fe²⁺ rather than Mn²⁺ is the natural activator of this enzyme (Snoko *et al.*, 1971; MacDonald & Lardy, 1978a).

It has been found that purified phosphoenolpyruvate carboxykinase does not respond to Fe²⁺ and that liver cytosol contains a protein that permits

Fe²⁺ to activate the enzyme (Bentle *et al.*, 1976). The new protein has been isolated in pure form (Bentle & Lardy, 1977) and named ferroactivator. A possible mechanism for rapid control of phosphoenolpyruvate carboxykinase *in vivo* involves inhibition by micromolar concentrations of P_i in the presence of Fe²⁺ (Bentle & Lardy, 1976) and activation by Fe²⁺ and ferroactivator protein (Bentle *et al.*, 1976; Bentle & Lardy, 1977). Conversion of ferroactivator from a latent into an active form under the influence of catecholamines or glucagon and from an active into a latent form by insulin is a possible mechanism for regulating rates of gluconeogenesis.

Blank *et al.* (1974) reported that 3-aminopicolinic acid causes hyperglycaemia in the intact rat. In view of its close structural similarity to quinolinic acid and to 3-mercaptopicolinic acid, which inhibit phosphoenolpyruvate carboxykinase (Foster *et al.*, 1966, 1967; Ray *et al.*, 1966; Veneziale *et al.*, 1967;

Di Tullio *et al.*, 1974; Kostos *et al.*, 1975; Goodman, 1975; Blackshear *et al.*, 1975; Jomain-Baum *et al.*, 1976), we tested the effect of 3-aminopicolinate on the activity of this enzyme. It permitted Fe^{2+} to activate purified phosphoenolpyruvate carboxykinase 3–4-fold (MacDonald & Lardy, 1978a). Of more than 30 pyridinecarboxylate compounds tested, only 3-aminopicolinate enhanced the enzyme's activity (MacDonald & Lardy, 1978b). These results suggested that 3-aminopicolinate might be a model for ferro-activator. If the hyperglycaemia caused by 3-aminopicolinate is due to activation of phosphoenolpyruvate carboxykinase, this might be evidence that the availability of Fe^{2+} is a means of regulating the activity of this enzyme *in vivo*. The purpose of the present work was to determine whether the effects of 3-aminopicolinate on carbon flux and liver gluconeogenic intermediates in the intact animal were consistent with its hyperglycaemic effect being the result of an activation of phosphoenolpyruvate carboxykinase.

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Experimental

Animals

Sprague-Dawley rats weighing 200–250 g were used.

Materials

Radioisotopically labelled compounds were from Amersham/Searle, Chicago, IL, U.S.A. All auxiliary enzymes used in the enzyme assays were from Boehringer Mannheim, Indianapolis, IN, U.S.A. Pure pig insulin and pure pig glucagon, free of insulin, were generously given by Dr. Ronald Chance and Dr. William Bromer respectively, of Eli Lilly, Indianapolis, IN, U.S.A. The sources of almost all other materials were mentioned previously (MacDonald & Lardy, 1978a). If not, they were from P-L Biochemicals, Milwaukee, WI, U.S.A., Boehringer Mannheim, or Sigma Chemical Co., St. Louis, MO, U.S.A., in the highest purity available.

Enzyme assays

Activities of all enzymes were assayed by the methods cited: phosphoenolpyruvate carboxykinase in crude rat liver cytosol by a modification (Bentle & Lardy, 1976) of the method of Seubert & Huth (1965), rat liver mitochondrial pyruvate carboxylase (McClure *et al.*, 1971), pig heart cytoplasmic aspartate aminotransferase (from Boehringer Mannheim) (Fahien & Strmecki, 1969), phosphoglycerate kinase

(Krietsch & Bücher, 1970), glyceraldehyde phosphate dehydrogenase, aldolase and pyruvate kinase (Bergmeyer & Gawehn, 1974) (the last four activities were assayed in crude rat liver cytosol). Purified pig kidney fructose bisphosphatase was kindly assayed by Dr. Frank Marcus (Marcus, 1975). Rat liver glucose 6-phosphatase activity in microsomal fractions and in deoxycholate extracts of microsomal fractions was assayed by the procedure of Nordlie & Arion (1966). Rat liver cytosol was a supernatant fraction obtained by centrifuging a 1:3 (w/v) homogenate of rat liver in 0.25 M-sucrose and 0.01 M-triethanolamine (Cl^-), pH 7.5, at 105000 g for 1 h.

Methods of analysis

To prepare liver for metabolite analysis, rats were stunned with a blow to the head and hemisected at the level of the diaphragm with a guillotine. Within 10 s of stunning, the liver was removed and pulverized to a fine powder in a tissue press previously chilled in liquid N_2 . A portion of the pulverized liver (3–4 g) was homogenized in 7–8 ml of 6% (w/v) perchloric acid and centrifuged at 15000 g for 5 min and the pellet discarded. A portion of the acid extract was mixed with acid-washed, activated charcoal (Norit-A) (8 mg/ml) for 10 min (Lowry & Passonneau, 1972) and neutralized with 30% (w/v) KOH. The rest of the acid extract was directly neutralized with KOH. The precipitates were removed by centrifugation, and the charcoal-treated extract filtered through Whatman no. 1 filter paper to remove charcoal that was not pelleted by centrifugation. All metabolites were measured spectrophotometrically by established enzymic methods (Bergmeyer & Gawehn, 1974). The charcoal-treated extract was used for assays requiring NADH (oxaloacetate, pyruvate, acetoacetate, phosphoenolpyruvate, 2-phosphoglycerate, 3-phosphoglycerate, citrate and aspartate) to eliminate the non-specific drift in absorbance encountered in these assays. It was previously determined that treatment with this small amount of charcoal removes less than 5% of these metabolites.

Glucose was determined by the glucose oxidase method (Huggett & Nixon, 1957) with a kit from Boehringer Mannheim Co. Glycogen was determined by the anthrone method (Good *et al.*, 1933; Seiftes *et al.*, 1950). Serum insulin was determined by a double-antibody radioimmunoassay (Soeldner & Slone, 1965), with pig insulin as a standard.

Radioisotopic glucose experiments in the intact rat

Polyethylene catheters (PE 10 and PE 50; Clay-Adams, Parsippany, NJ, U.S.A.) were inserted via the common carotid artery and external jugular vein into the aorta and vena cava respectively of anaesthetized rats through a 2 cm neck incision 1 week before

experimentation as described by Popovic & Popovic (1960). Experiments were carried out on conscious, unanaesthetized rats kept in individual cages to restrain movement. Animals were placed in the cages 1 h before the experiments were begun and remained at ease throughout the experimental period. Radioactive isotopes were infused into the venous catheter. Blood samples (0.1 ml) were obtained from the arterial catheter at 10 min intervals, and the amount withdrawn was replaced with 0.9% NaCl. Blood was immediately placed in 1 ml of 6% (w/v) perchloric acid at 0°C. Precipitated protein was removed by centrifugation.

Isolation of radioactive blood glucose

Unlabelled 30% glucose in water (0.3 ml) was added to 0.2 ml of the perchloric acid extract. The glucose was converted into gluconic acid by the addition of 7 ml of 3.6% (w/v) methanolic I_2 and warming to 50°C for 10 min. The gluconic acid was precipitated as the potassium salt by the addition of 5 ml of 5% (w/v) methanolic KOH (Andrews *et al.*, 1965). The precipitate was allowed to settle for 18 h and then washed three times with methanol. It was dried under reduced pressure at 50°C, weighed and dissolved in 1.2 ml of water. This solution was mixed with 10 ml of a 2:1 (v/v) mixture of toluene and Triton X-100 containing 0.27% (w/v) PPO (2,5-diphenyloxazole) and 0.0067% POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]. The radioactivity of this mixture was determined in an Isocap 300 (Amersham/Searle Co.) liquid-scintillation spectrometer.

Statistical methods

Slopes of lines were determined by linear-regression analysis. Walsh's modification (Snedecor & Cochran, 1967) of Student's *t* test was used to determine whether slopes of lines were significantly different. Student's ordinary *t* test was used for other comparisons.

Results

Influence of 3-aminopicolinate on blood glucose

The present study confirms the report of Blank *et al.* (1974) that 3-aminopicolinate causes hyperglycaemia in the intact rat (Fig. 1). It is well established that the liver glycogen content of the starved rat is very low (Korec, 1967). The liver glycogen concentration of the 24 h-starved rats used in the present study was found to be 2.9 ± 1.4 ($n = 3$) as against $348 \pm 30 \mu\text{mol}$ of glucose equiv./g of liver in the fed rat ($n = 3$). Thus, even if all the liver glycogen of the starved rat were converted into glucose, it would account for only a small fraction of the increase in blood glucose observed as a consequence of 3-aminopicolinate injection.

Fig. 1 also shows that 3-aminopicolinate does not cause hyperglycaemia via glucagon release. The amount of glucagon administered to the rats (1 mg), which is enough to increase the blood glucose of an adult human, caused a prompt increase in the blood glucose, but only to the extent of 20%. This is in contrast with the progressive and extensive increase after administration of 3-aminopicolinate.

Rats treated with 3-aminopicolinate have very high concentrations of serum insulin (Table 1), probably as a result of hyperglycaemia. This rules out the possibility that 3-aminopicolinate causes hyperglycaemia by inhibiting insulin release. Decreased liver acetoacetate and β -hydroxybutyrate concentrations of the rats treated with 3-aminopicolinate also indicate that insulin concentrations were increased. The mean (± 1 s.d.) combined acetoacetate and β -hydroxybutyrate concentrations were 1.3 ± 0.3 and $0.6 \pm 0.1 \mu\text{mol/g}$ of liver in the 24-h-starved rats

Fig. 1. Effect of 3-aminopicolinate on the blood glucose of rats

3-Aminopicolinate, pH 7.4 (100 mg/kg body wt.), was injected into the indwelling arterial catheters of alert, unanaesthetized 24 h-starved rats at 60 min ($\bullet$). The effects of glucagon (1 mg/rat) injected intraperitoneally (one rat) or intravenously (one rat) at zero time, or infused intravenously from 0 to 60 min (three rats), are shown only for comparison (Δ). Results with glucagon were averaged, since the magnitude of the effects with all three routes of administration were similar. Control rats were given 0.9% NaCl intravenously at 60 min (seven rats, $\circ$). During the period 0–60 min the mean glucose concentrations in the control rats (not shown for the sake of clarity) were similar to those of the 3-aminopicolinate-treated rats before treatment. A sample (0.1 ml) of blood was withdrawn from the arterial catheter and immediately placed in 1.0 ml of cold 6% perchloric acid and replaced with heparinized 0.9% NaCl at 10-min intervals. The mean glucose concentrations (± 1 s.e.m.) are shown.

Table 1. *Effect of 3-aminopicolinate on serum insulin concentrations in the 24h-starved rat*

Serum insulin was determined 90min after an intravenous injection of 3-aminopicolinate (100mg/kg body wt.) or 0.9% NaCl. The number of rats in each group is shown in parentheses.

Condition of Rats	Serum insulin (μ units/ml)
Fed (4)	$30 \pm 9^*$
Starved 24h + NaCl (5)	9 ± 15
Starved 24h + 3-aminopicolinate (6)	$>139 \pm 25^\dagger$

* Mean $\pm$ S.D.

† This represents the minimum average. Three of the values were $>160 \mu$ units/ml, the upper limit of the assay. For these three values, a value of 160μ units of insulin/ml was used to calculate the mean $\pm$ S.D.

($n = 8$) and the rats treated with 300mg of 3-aminopicolinate/kg body wt. intraperitoneally for 1h ($n = 4$) respectively.

Effect of 3-aminopicolinate on production and utilization of labelled glucose

In rats given $[6\text{-}^3\text{H}]\text{glucose}$ intravenously, the rate of decrease of the blood $[6\text{-}^3\text{H}]\text{glucose}$ concentration was not altered by 3-aminopicolinate, indicating that the hyperglycaemia associated with 3-aminopicolinate was not due to inhibition of glucose utilization or a redistribution of total body glucose. The more rapid decrease in blood $[6\text{-}^3\text{H}]\text{glucose}$ specific radioactivity after 3-aminopicolinate administration resulted from dilution of the existing glucose pool by newly synthesized glucose (Fig. 2). If the major effect had been rather an inhibition of glucose utilization, the rate of decrease in the total blood glucose radioactivity would have slowed, and the rate of decrease in specific radioactivity would not have been altered. Feller *et al.* (1950) showed that in alloxan-diabetes in the rat, which is an insulin-deficient state, enhanced gluconeogenesis rather than impaired glucose utilization is the main cause of hyperglycaemia. The results of the present study (Figs. 2–5) are in agreement with the hypothesis that the hypoglycaemic effect of insulin is mediated more by suppression of gluconeogenesis than by stimulation of glucose utilization (Ui *et al.*, 1975), because any hypoglycaemic effects of the high concentrations of circulating insulin, besides effects on gluconeogenesis, in the 3-aminopicolinate-treated rats were inadequate to prevent the steady increase in their blood glucose.

Administering 3-aminopicolinate enhanced the rates of $[^{14}\text{C}]\text{alanine}$ and $[^{14}\text{C}]\text{lactate}$ incorporation into glucose (Figs. 3 and 4), but had either no effect on, or slightly inhibited, the rate of incorporation of $[^{14}\text{C}]\text{glycerol}$ and $[^{14}\text{C}]\text{xylitol}$ into glucose (Fig. 5).

Fig. 2. *Effect of 3-aminopicolinate on the disappearance of total and specific radioactivity of blood $[6\text{-}^3\text{H}]\text{glucose}$ in the intact rat*

$[6\text{-}^3\text{H}]\text{Glucose}$ (sp. radioactivity $1 \text{ mCi}/\mu\text{mol}$ in 0.1 ml of 0.9% NaCl; $50 \text{ mCi}/\text{rat}$) was injected into the indwelling venous catheters of alert, unanaesthetized 24h-starved rats at zero time, and 3-aminopicolinate, $\text{pH } 7.4$ (100 mg in 1 ml of water/kg body wt.) (circles) or 0.9% NaCl (triangles) was injected into the arterial catheters after a 60 min control period (indicated by a vertical arrow). The rats receiving NaCl served as external controls. A sample (0.1 ml) of blood was withdrawn from the arterial catheter and immediately placed in 1.0 ml of cold 6% perchloric acid and replaced with heparinized 0.9% NaCl at 10-min intervals. The total radioactivity ($\bullet$, $\blacktriangle$) and specific radioactivity ($\circ$, $\triangle$) of blood $[6\text{-}^3\text{H}]\text{glucose}$ were plotted on a logarithmic scale against time. The isolation of glucose and the determination of radioactivity is described in the Experimental section. Results are the average values from five rats treated with 3-aminopicolinate and two rats treated with NaCl. Linear-regression analysis was used to fit a line to the data points from 10 to 180 min for the NaCl-treated rats, and one line from 10 to 60 min and another line from 70 to 180 min for the 3-aminopicolinate-treated rats. The slope of the specific radioactivity during 3-aminopicolinate treatment was significantly different ($P < 0.01$) from that of the control period and from that of the rats treated with NaCl. Slopes of total radioactivity were not different.

This is consistent with an activation in the gluconeogenesis pathway between pyruvate and the triose phosphates. By comparing the pretreatment and post-treatment slopes (calculated by linear-regression analysis) of radioactivity in blood glucose, it was

Fig. 3. Effect of 3-aminopicolinate on the incorporation of carbon from [^{14}C]alanine into the blood glucose in the intact rat

[U- ^{14}C]Alanine (sp. radioactivity $164\mu\text{Ci}/\mu\text{mol}$) was infused via the indwelling venous catheter at a constant rate of $0.2\mu\text{Ci}/\text{min}$ throughout the time period shown. 3-Aminopicolinate (five rats, $\bullet$) or 0.9% NaCl (three rats, $\circ$) was injected into the arterial catheter after a 60 min control period (indicated by a vertical arrow). The rats given only NaCl served as external controls. The condition of the rats, doses of 3-aminopicolinate and NaCl and blood-sampling procedures were as described in Fig. 2. Linear-regression analysis was used to fit a line to the data points from 10 to 120 min for the control rats; and one line for points from 10 to 60 min and another line for points from 70 to 150 min for the 3-aminopicolinate-treated rats. Data points are the average values for each group at each time interval. The slope during 3-aminopicolinate treatment was significantly different ($P < 0.0005$) from that of either type of control period.

determined that the mean net ^{14}C incorporation into glucose from ^{14}C -labelled alanine and lactate was increased 90% ($P < 0.0005$, $n = 5$) and 40% ($P < 0.002$, $n = 5$) respectively. The somewhat decreased incorporation of glycerol and xylitol into glucose after 3-aminopicolinate injection could have been due to dilution of the radioactively labelled carbon pool by unlabelled gluconeogenic precursors that enter the gluconeogenesis pathway before the triose phosphates.

Influence of 3-aminopicolinate on liver gluconeogenic intermediates

Fig. 6 shows that the predominant effect of 3-aminopicolinate on the liver metabolite pattern of the 24h-starved rat was to increase phosphoenolpyruvate to nearly 7-fold the normal value. The activities of rat liver phosphoglycerate kinase, pyruvate kinase, glyceraldehyde phosphate dehydrogenase and aldolase were not inhibited when assayed directly in the presence of 1.2 mM-3-aminopicolinate with or with-

Fig. 4. Effect of 3-aminopicolinate on the incorporation of carbon from [^{14}C]lactate into the blood glucose in the intact rat

[U- ^{14}C]Lactate (sp. radioactivity $50\mu\text{Ci}/\mu\text{mol}$) was infused via the indwelling venous catheter at a constant rate of $0.2\mu\text{Ci}/\text{min}$ throughout the time period shown. 3-Aminopicolinate was injected into the arterial catheter after a 60 min control period (indicated by the arrow). A representative experiment is shown in which the slope after 3-aminopicolinate treatment was 1.6 times that before it was administered. Linear-regression analysis was used to fit two lines to the data points, one line from points at 10 to 60 min and the other line for points from 70 to 170 min. The average of the slopes from five rats after 3-aminopicolinate treatment was 1.4 times that of the control period. The difference was significant in each of the five experiments ($P < 0.002$). For two control rats, the average slope from 70 to 170 min was not significantly different from 0 to 60 min. These are not shown as the d.p.m./ml of blood of the two groups were different, but the slopes were parallel to the control period of the 3-aminopicolinate-treated rats. The conditions of the rat, dose of 3-aminopicolinate, blood sampling and analysis procedures were the same as described in Fig. 2.

out $400\mu\text{M-FeCl}_2$. This is consistent with an activation of gluconeogenesis at the step catalysed by phosphoenolpyruvate carboxykinase, rather than an inhibition of a subsequent enzyme of the gluconeogenesis pathway.

In addition to the increase in phosphoenolpyruvate, liver malate, citrate, aspartate, alanine, lactate and pyruvate were increased with 3-aminopicolinate treatment. Except for malate, they were not increased as much as was phosphoenolpyruvate (Fig. 6). Hsu & Fahien (1976) have reported that quinolinate, a structural analogue of 3-aminopicolinate, inhibits cytosolic aspartate aminotransferase competitively with respect to 2-oxoglutarate ($K_i = 0.2\text{mM}$). Although the effects of 3-aminopicolinate on the liver malate, citrate and aspartate were minor, compared

Fig. 5. Effect of 3-aminopicolinate on the incorporation of carbon from [^{14}C]glycerol and [^{14}C]xylitol into the blood glucose in the intact rat

[U- ^{14}C]Glycerol (sp. radioactivity $9.6\mu\text{Ci}/\mu\text{mol}$) or [U- ^{14}C]xylitol (sp. radioactivity $3.2\mu\text{Ci}/\mu\text{mol}$) was infused via the indwelling venous catheter at a constant rate of $0.15\mu\text{Ci}/\text{min}$ or $0.11\mu\text{Ci}/\text{min}$ respectively throughout the time period shown. After a 60min control period, 3-aminopicolinate (three rats, ●) or NaCl (two rats, ○) was injected into the arterial catheter of the rats receiving glycerol and 3-aminopicolinate was injected into two rats receiving xylitol (■). The time of injection is indicated by the arrow. The condition of the rats, dose of 3-aminopicolinate or NaCl, blood sampling and analysis procedures were the same as described in Fig. 2. Each point is the mean $\pm$ s.d. Linear-regression analysis of the data from xylitol-treated rats was used to fit two lines to the data points, one line from points at 10 to 60min and the other line for points from 70 to 170min. Data points are the average values.

with the 10–35-fold increases obtained with quinolinate administration to the intact rat (Ray *et al.*, 1966), the question was asked whether the inhibition of this enzyme might be responsible for the increased concentrations of these metabolites. When it was assayed in the presence of 2-oxoglutarate as low as $50\mu\text{M}$, 3-aminopicolinate (1.2mM) did not inhibit aspartate aminotransferase. Quinolinate did inhibit the enzyme, thus confirming the results of Hsu & Fahien (1976).

The increases in malate, citrate, aspartate, alanine, lactate and pyruvate were not as great, relative to those of phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate when they were determined after only 10min of treatment with a lower dose of 3-aminopicolinate (Fig. 7), indicating that this metabolite pattern may have been a secondary or a separate minor effect of 3-aminopicolinate. Since the former metabolites have been reported to be variously increased in the livers of animals in the fed state (Bergmeyer & Gawehn, pp. 2266–2302, 1974; MacDonald *et al.*, 1976), in diabetes (Kalkhoff *et al.*,

Fig. 6. Effect of 3-aminopicolinate on liver gluconeogenic intermediates of the intact rat after 60min treatment. Metabolites were determined 60min after 150mg (●, eight rats) or 300mg (○, six rats) of 3-aminopicolinate per kg body wt. was injected intraperitoneally to rats starved for 24h. Values are expressed as a percentage ($\pm$ s.d.) of the average concentrations in livers from control rats ($n = 12$). The latter are shown at the lower margin of the Figure, and the s.d. of these values, expressed as percentages, are shown by the vertical lines placed along the line equal to 100%. Abbreviations used: MAL, malate; CIT, citrate; ASP, aspartate; GLU, glutamate; ALA, alanine; LAC, lactate; PYR, pyruvate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; G, glucose.

Fig. 7. Effect of 3-aminopicolinate and glucose administration on liver gluconeogenic intermediates of the intact rat 10min after treatment

Metabolites were determined 10min after 3-aminopicolinate ($50\text{mg}/\text{kg}$ body wt., four rats, ●), or glucose ($200\text{mg}/\text{kg}$ body wt., eight rats, ○), were injected intravenously into rats starved for 24h. Values are expressed as described in the legend to Fig. 2. There were eight control rats.

Table 2. Effect of 3-aminopicolinate on liver adenine nucleotides, fructose bisphosphate and dihydroxyacetone phosphate. Metabolites were determined 60 min after an intraperitoneal injection of 3-aminopicolinate (150 mg/kg body wt.) was administered to 24 h-starved rats. Control rats were starved for 24 h. The number of rats in each group is shown in parentheses.

Metabolite	Concn. of metabolite ($\mu\text{mol/g}$ of liver)	
	Control (6)	3-Aminopicolinate (4)
ATP	2.7 $\pm$ 0.4*	3.2 $\pm$ 0.5*
ADP	1.2 $\pm$ 0.5	1.6 $\pm$ 0.2
AMP	0.21 $\pm$ 0.06	0.22 $\pm$ 0.07
Fructose bisphosphate	0.024 $\pm$ 0.005	0.040 $\pm$ 0.015
Dihydroxyacetone phosphate	0.032 $\pm$ 0.002	0.056 $\pm$ 0.016

* Mean $\pm$ S.D.

1966), by feeding diets high in glucose or fructose (Zakim *et al.*, 1967), by injections of fructose (Burch *et al.*, 1969) or lactate (Hornbrook *et al.*, 1965) and by injecting glucagon (Williamson, 1967), it was suspected that the increases in these metabolites may have been a result of the hyperglycaemia induced by 3-aminopicolinate. Fig. 7 shows that liver malate, alanine, lactate and pyruvate concentrations in the starved rat were increased, 10 min after a glucose injection, in a pattern similar to that obtained 10 min after an injection of 3-aminopicolinate, but that phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate were not increased by the glucose injection. An additional experiment (results not shown) indicated that malate, citrate, aspartate, lactate, alanine and pyruvate were increased to 222, 469, 214, 307 and 156% respectively of their control values, after a 1 h infusion of glucose (1.2 g/h per kg) demonstrating that, with prolonged treatment, these alterations could be as pronounced as with prolonged treatment with 3-aminopicolinate. Phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate were not increased more than 30% by the 1 h infusion of glucose. The infusion of glucose did not mimic the 3-aminopicolinate injections in regard to glutamate, as the glutamate concentrations were consistently increased with glucose administration, as in Fig. 7, but were consistently normal or low after 3-aminopicolinate administration. Thus hyperglucosaemia probably only partially explains the altered concentrations of metabolites, other than the phosphorylated C₃ acids, associated with 3-aminopicolinate administration.

Fructose 6-phosphate and glucose 6-phosphate were decreased by 3-aminopicolinate treatment (Figs. 6 and 7), and fructose bisphosphate was slightly increased (Table 2), indicating an apparent inhibition of fructose bisphosphatase or activation of glucose 6-phosphatase. When assayed directly in the presence of 1.2 mM-3-aminopicolinate, fructose bisphosphatase was not inhibited and glucose 6-phosphatase was not activated. Therefore any effect of

3-aminopicolinate on these enzymes must have been indirect. It is well known that ATP and AMP are effectors of fructose bisphosphatase and phosphofructokinase. However, liver nucleotide concentrations (Table 2) were not significantly altered by 3-aminopicolinate administration. This is in agreement with the fact that 3-aminopicolinate did not alter O₂ consumption by isolated mitochondria (metabolizing succinate or malate) unless the concentration was 10 mM or greater (results not shown). The highest concentration of 3-aminopicolinate found in rat liver after the intraperitoneal administration of 300 mg/kg body wt. was less than 1 $\mu\text{mol/g}$ of liver (results not shown). There is evidence that insulin can modulate the kinetic properties of fructose bisphosphatase and/or phosphofructokinase (Taunton *et al.*, 1974; Williamson, 1967). The apparent inhibition of fructose bisphosphatase could be related to the hyperinsulinism associated with 3-aminopicolinate treatment.

Discussion

In the present study we have extended the findings of Blank *et al.* (1974) by showing that the hyperglycaemia caused by 3-aminopicolinate (Fig. 1) is not the result of glycogenolysis, for the liver glycogen concentrations were too low to account for the glucose formed. 3-Aminopicolinate did not cause hyperglycaemia by the inhibition of insulin release; rather, it caused hyperinsulinaemia (Table 1). 3-Aminopicolinate did not significantly interfere with [6-³H]glucose utilization in the intact rat and it caused a dilution of the existing [6-³H]glucose pool with newly synthesized glucose (Fig. 2). The incorporation of [¹⁴C]alanine and [¹⁴C]lactate into glucose was increased (Figs. 3 and 4), whereas that from [¹⁴C]glycerol and [¹⁴C]xylitol was unchanged or slightly decreased (Fig. 5). Since alanine and lactate enter the gluconeogenic pathway as pyruvate, and glycerol and xylitol (Williamson *et al.*, 1971) enter the pathway beyond this point at the level of the

triose phosphates or fructose 6-phosphate, this indicates that gluconeogenesis was activated at a step between pyruvate and the triose phosphates. A predominant effect of 3-aminopicolinate on the liver gluconeogenic-intermediate patterns was to increase the phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate concentrations (Figs. 6 and 7). This pattern is consistent with a stimulation of gluconeogenesis at or near the step catalysed by phosphoenolpyruvate carboxykinase.

Liver citrate, aspartate, alanine, lactate and pyruvate were increased after 1 h of 3-aminopicolinate treatment, but not as much as was phosphoenolpyruvate. Malate was increased as much as phosphoenolpyruvate (Fig. 6). Increases in these metabolites were not as great, relative to phosphoenolpyruvate, when they were determined 10 min after a low dose of 3-aminopicolinate (Fig. 7), suggesting that this pattern could have been a secondary or a separate effect of 3-aminopicolinate.

Since it is well established that liver alanine, lactate, and pyruvate concentrations are 2–3-fold higher in the fed state than in the starved state (Bergmeyer & Gawehn, pp. 2266–2302, 1974; MacDonald *et al.*, 1976), it was suspected that the increases in these metabolites may have been a result of the hyperglycaemia induced by 3-aminopicolinate. It has also been reported that the concentrations of liver malate, citrate and aspartate are increased in diabetic rats (Kalkhoff *et al.*, 1966) or when diets high in fructose or glucose (Zakim *et al.*, 1967) and injections of fructose (Burch *et al.*, 1969) or lactate (Hornbrook *et al.*, 1965) are administered. The data of Williamson (1967) show that malate is increased as much as phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate with the administration of glucagon and anti-insulin serum to the intact rat, whereas the data of Exton & Park (1969) and Blair *et al.* (1973) show that when glucagon is added to the perfused liver phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate, but not malate, are increased. This suggests that although a primary effect of glucagon is at the step catalysed by phosphoenolpyruvate carboxykinase, not unexpectedly, the pattern of liver gluconeogenic intermediates is influenced by other organs and by the hormonal milieu of the intact animal. A similar argument can be used to explain the liver metabolite pattern observed with 3-aminopicolinate. Injecting high and low doses of glucose into intact rats produced increases in liver malate, citrate, aspartate, lactate, alanine and pyruvate (Fig. 7 and the Results section), but no increases in phosphorylated C₃ acids were observed. Other possible metabolic effects of 3-aminopicolinate, relevant to the increased concentrations of non-phosphorylated metabolites, require investigation.

We showed previously (MacDonald & Lardy, 1978a) that 3-aminopicolinate is a model for the

naturally occurring ferroactivator protein that permits Fe²⁺ to activate purified phosphoenolpyruvate carboxykinase. If, as the present work suggests, activation of phosphoenolpyruvate carboxykinase is the mechanism by which 3-aminopicolinate causes hyperglycaemia, this would allow the following postulations. (1) Liver phosphoenolpyruvate carboxykinase is normally not maximally active *in vivo*, since it can be activated. Activation *in vivo* could be mediated by ferroactivator and Fe²⁺ (Bentle *et al.*, 1976; Bentle & Lardy, 1977). (2) Enough iron can be present as Fe²⁺ in liver to activate this enzyme. The second postulation requires some discussion. There are no free transition-metal ions in liver, since they are present in only micromolar amounts and are avidly chelated by numerous molecules including proteins, amino acids, sugars, P_i and nucleotides. It is known that ferric iron is tightly bound to ferritin, a major storage site of iron in liver, and that it must be reduced to Fe²⁺ to be released from ferritin *in vitro* (Davis *et al.*, 1962). Frieden and his co-workers (cf. Frieden & Osaki, 1974) have discovered a mechanism by which Fe³⁺ bound to ferritin can be reduced and released. The ferroactivator protein could function by binding the released Fe²⁺ and transferring it to the active site of phosphoenolpyruvate carboxykinase. If 3-aminopicolinate, which does not activate purified phosphoenolpyruvate carboxykinase in the absence of a bivalent transition-metal ion *in vitro* (MacDonald & Lardy, 1978a), does activate phosphoenolpyruvate carboxykinase *in vivo*, it must do so by making a bivalent transition metal available to the enzyme, in effect substituting for the ferroactivator. Since iron is the most prevalent transition metal in liver, Fe²⁺ would most likely be the activating metal. The cytosolic concentration of iron (redox state unknown) in liver is about 186 µmol/kg, whereas those of manganese (1.8 µmol/kg) (Thiers & Vallee, 1957) and cobalt (<1 µmol/kg) (Bowen, 1966), the other two bivalent metal ions that 3-aminopicolinate permits to activate purified phosphoenolpyruvate carboxykinase, are lower than that of the enzyme (about 3 µmol/kg). Since ferroactivator can permit as low as 1–5 µM-Fe²⁺ to give nearly maximal activation of the enzyme *in vitro* (Snoke *et al.*, 1971; Bentle & Lardy, 1977), very little iron in liver would need to be present as Fe²⁺ for activation of phosphoenolpyruvate carboxykinase. The possibility that, in the experiments with 3-aminopicolinate, the activating metal ion is derived from an extrahepatic source, cannot be ruled out, but since iron is the most abundant transition metal in the body, Fe²⁺ would still be the most likely metal ion to activate the enzyme.

It is noteworthy that Friedmann & Rasmussen (1970) reported that 0.05 mM-Mn²⁺ stimulates glucose synthesis from lactate by the perfused liver, and Papavasiliou *et al.* (1968) have shown that Mn²⁺

causes hyperglycaemia when administered to the intact rat. These effects may very well be the result of activating phosphoenolpyruvate carboxykinase in the liver, for Mn^{2+} is a potent activator of purified phosphoenolpyruvate carboxykinase (Bentle & Lardy, 1976, 1977; MacDonald & Lardy, 1978a) and overcomes quinolinate-induced inhibition of glucose production in perfused liver (Veneziale *et al.*, 1967).

Speculation

In the rat, insulin deficiency induced either with alloxan or mannoheptulose or by pancreatectomy increases the liver content of phosphoenolpyruvate carboxykinase, and insulin treatment of the alloxan-diabetic rat restores the enzyme activity to the normal value (Shrago *et al.*, 1963; Young *et al.*, 1964). There is also evidence that insulin can modulate the kinetic properties of phosphoenolpyruvate carboxykinase, but it is scant and indirect (Exton & Park, 1969; Williamson, 1967; Ui *et al.*, 1973). The high insulin concentrations caused by 3-aminopicolinate clearly show that this compound causes insulin resistance. The site(s) at which 3-aminopicolinate acts must be an important regulatory site of insulin, since the high concentration of insulin acting at other sites was inadequate to lower the blood glucose. One may speculate that if 3-aminopicolinate causes hyperglycaemia by activating phosphoenolpyruvate carboxykinase, the step catalysed by this enzyme is an important site of insulin action.

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