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# POTENTIATION OF BENZOATE TOXICITY BY GLYOXYLATE

## INHIBITION OF PYRUVATE CARBOXYLASE AND THE UREA CYCLE*

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**Abstract**—It has been proposed that administration of non-nitrogenous precursors to glycine is necessary to realize the full potential of benzoate metabolism as a pathway for disposal of waste nitrogen during ammonia intoxication (Coude *et al.*, *Clin Chim Acta* **136**: 211–217, 1984). However, when glyoxylate, a keto acid precursor to glycine, was administered with benzoate 1 hr prior to a challenge of ammonia, protection against ammonia toxicity was less successful than with benzoate alone. At the cellular and subcellular levels, glyoxylate and benzoate each inhibited the urea cycle in isolated hepatocytes and pyruvate carboxylase in isolated mitochondria. The action of each drug was associated with depletion of aspartate content in isolated hepatocytes and reduction of pyruvate-dependent incorporation of $CO_2$ into aspartate in assays with isolated mitochondria. Depression of aspartate regeneration by inhibition of pyruvate carboxylase is a likely mechanism for impairment of urea cycle activity by both drugs. In whole animals, inhibition of pyruvate carboxylase may contribute to benzoate toxicity and the adverse influence of glyoxylate on benzoate therapy.

The promise of benzoate therapy to combat ammonia toxicity rests with the capacity of the drug to promote the flow of waste nitrogen into the glycine moiety of its metabolic end product, hippurate [1]. Since glycine is limiting in the conversion of benzoate to hippurate, non-nitrogenous precursors could augment disposal of waste nitrogen by this route. Piridoxilate, a European cardiac drug, stimulated hippurate synthesis 10-fold in suspensions of isolated hepatocytes, presumably because the drug is cleaved to pyridoxine and glyoxylate, a facile keto acid precursor to glycine [2]. Based on this result, the administration of piridoxilate was recommended to improve the effectiveness of benzoate therapy against ammonia intoxication.

At about four times its therapeutic dose benzoate potentiates ammonia toxicity, apparently by inhibiting the urea cycle through depletion of acetyl CoA [3–5]. Pyruvate carboxylase (EC 6.4.1.1), a major source of the carbon chain for regeneration of aspartate, requires acetyl CoA as an activator and has been implicated as a possible site of inhibition [4, 5]. Piridoxilate has the potential to intensify adverse reaction to benzoate because its metabolite, glyoxylate, is a structural analogue of pyruvate that could also inhibit pyruvate carboxylase, either directly [6] or through its conversion to oxalate [7, 8]. Results reported below show that, when these proposed interactions were tested, glyoxylate compromised protection by benzoate against a challenge of ammonia, glyoxylate and benzoate each depleted aspartate and inhibited the urea cycle in suspensions of isolated hepatocytes, and each drug reduced pyruvate-dependent incorporation of bicarbonate into aspartate in suspensions of isolated mitochondria.

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### MATERIALS AND METHODS

Radioisotopes and Aquasol (liquid scintillation fluid) were obtained from New England Nuclear (Boston, MA). Inorganic chemicals were purchased from Fisher Scientific (Medford, MA). All other chemicals and enzymes were obtained from the Sigma Chemical Co. (St Louis, MO). Male Sprague–Dawley rats (275–350 g) from the Charles River Colony (Wilmington, MA), were used throughout. Hepatocytes were isolated by perfusion of the liver with collagenase, according to Seglen [9]. Conditions for measurement of ureagenesis and hippurate synthesis were as described previously [3] except that urea was determined colorimetrically [10]. Urease-labile chromogenic product was used to calculate urea formation.

In assays with isolated mitochondria, rats fasted for 18 hr were killed by decapitation; the liver of each rat was quickly excised and homogenized with a Teflon pestle in 10 vol. of ice-cold 0.25 M mannitol made 1 mM in EDTA and adjusted to pH 7.2. Mitochondria were isolated from the homogenate according to Myers and Slater [11]. The pellet obtained by centrifugation at 800 *g* for 5 min was discarded, and the supernatant fluid was centrifuged at 7000 *g* for 10 min. The resultant pellet was washed twice by resuspension in homogenizing medium and sedimentation at 18,000 *g* for 10 min. The washed mitochondrial pellet was resuspended in homogenizing medium at 20–30 mg protein/ml for assay. Protein was determined with biuret reagent.

Isolated mitochondria were incubated in the assay system of Stirk *et al.* [12] to determine pyruvate

carboxylase activity by measuring pyruvate-dependent incorporation of [$^{14}$C]$KHCO_3$ into acid-stable radiolabeled products. The reaction mixture consisted of KCl (111 mM), $K_2HPO_4$ (2.5 mM), sodium pyruvate (5 mM), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (10 mM), [$^{14}$C]$KHCO_3$ (0.3 to 7.5 $\mu$Ci, 20 mM) and mitochondrial suspension (200 $\mu$l, 4–6 mg protein) in a final volume of 2 ml at pH 7.6. Benzoate and glyoxylate were added, where indicated, as solutions made isotonic with KCl. Reaction mixtures were incubated in open scintillation vials at 30°, acidified at 10 min with 0.5 ml of 1.6 N HCl, and then baked to dryness over a steam bath. The residues so obtained were extracted with 2 ml water and the extracts diluted with 6.5 ml Aquasol to measure acid-stable radiolabeled product in a liquid scintillation counter. Measurements at 2.5-min intervals with and without inhibitors showed product accumulation to be linear for at least 12.5 min.

In assays of pyruvate carboxylase in particle-free extracts, washed mitochondria were lysed by resuspension in water to yield 40–60 mg protein/ml and sonication with three pulses of 20 sec each in an ice bath. Insoluble matter was removed by centrifugation at 18,000 *g* for 10 min. The supernatant fluid was diluted with 4 vol. of water for assay in 2 ml of the following composition: ATP (4 mM), HEPES buffer (pH 7.8, 0.1 M), $MgSO_4$ (12 mM), NADH (0.25 mM), malic dehydrogenase (EC 1.1.1.37, 20 units), [$^{14}$C]$KHCO_3$ (3 $\mu$Ci, 10 mM), sodium pyruvate and acetyl CoA (as indicated) and dilution of mitochondrial extract (100 $\mu$l). Product formation was measured as described above for assays with intact mitochondria except that the incubation period was reduced to 4 min. Measurements at 1, 2, 4 and 6 min showed product formation to be linear with and without inhibitors. Malate accumulation (see below) accounted for more than 90% of the $CO_2$ fixed under these assay conditions.

Methods for measurement of malate and aspartate were enzymatic. Malate content was determined by measuring absorbance at 340 nm owing to NADPH generation during incubation of the neutralized acid-soluble fraction of the reaction mixtures with malic enzyme (EC 1.1.1.40; 1.0 unit), Tris buffer (pH 7.4, 67 mM), $MgCl_2$ (4 mM), and NADP (0.5 mM) at room temperature in a reaction volume of 3 ml. Aspartate content of hepatocytes was determined similarly by measuring NADH oxidation in 3 ml of solution containing the neutralized acid-soluble fraction of harvested cells (30 *g* for 2 min), glutamic oxaloacetic transaminase (EC 2.6.1.1; 45 units), malic dehydrogenase (18 units), $\alpha$-ketoglutarate (5 mM), NADH (0.2 mM) and phosphate buffer (pH 7.2, 0.1 M). The reactions were allowed to proceed to equilibrium, and the change in absorbance was used to calculate malate and aspartate content. Analyses of authentic malate and aspartate by these procedures accounted for >85% of known quantities assayed, based on a 1:1 stoichiometry for change in reduced pyridine nucleotide content and metabolite concentration. Samples from reaction mixtures containing glyoxylate and benzoate did not interfere with the assays for authentic malate and aspartate.

When incorporation of [$^{14}$C]$KHCO_3$ into aspartate was measured, radiolabeled aspartate was isolated by cocrystallization with carrier from a particle-free dilution of the reaction mixture in 0.15 N HCl. Carrier aspartate was dissolved at 33 mg/ml with heat, and crystallization was initiated by addition of 0.67 vol. of ethanol. The product was recrystallized from 40% (v/v) ethanol to constant specific activity. Verification of the identity of the radiolabeled metabolite that cocrystallized with carrier was accomplished enzymatically. Prior treatment of the neutralized acid-soluble fraction with glutamic oxaloacetic transaminase and malic dehydrogenase under the conditions used to assay aspartate content (see above) resulted in loss of >92% of the radiolabeled metabolite that cocrystallized with carrier.

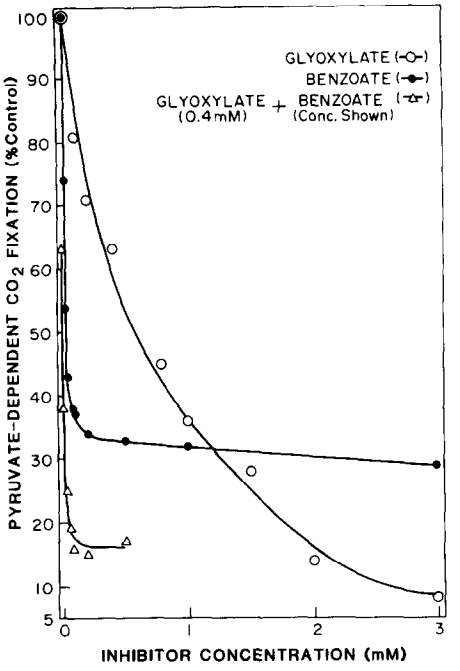


Fig. 1. Inhibition of pyruvate-dependent $CO_2$ fixation by glyoxylate and benzoate. Mitochondria (4–6 mg protein) were incubated at 30° for 10 min in 2-ml reaction mixtures containing [$^{14}$C]$KHCO_3$, (0.30 to 0.50 $\mu$Ci, 20 mM) and benzoate and glyoxylate at the concentrations indicated (see Materials and Methods for details).

Significance of differences between means was determined by analysis of variance with the Student–Neuman–Keuls multiple comparison test (Statistical Analysis Systems, Cary, NC) for data presented in the tables. Least-squares linear regression was used to determine linear fit of data presented in Figs. 2 and 3; correlation coefficients are >0.99.

## RESULTS AND DISCUSSION

Glyoxylate was substituted for piridoxilate, which is not available in the U.S., in a test to determine whether combination therapy with benzoate afforded greater protection against ammonia toxicity than therapy with benzoate alone. When groups of 13–20 fasted (18 hr) adult male rats were administered (i.p.) saline, benzoate (2.5 mmol/kg), or ben-

Table 1. Effects of glyoxylate and benzoate on ureagenesis, hippurate synthesis and aspartate content in isolated hepatocytes*

| Additions | Urea synthesis | Hippurate synthesis | Aspartate content |
|---|---|---|---|
| None (control) | 150 ± 6[a] | | 1.60 ± 0.06[a] |
| Glyoxylate (1 mM) | 79 ± 6[b] | | 0.27 ± 0.09[b] |
| Benzoate (1 mM) | 77 ± 5[b] | 2.5 ± 0.11[a] | 0.33 ± 0.09[b] |
| Glycine (1 mM) | 149 ± 9[a] | | 1.90 ± 0.16[a] |
| Glyoxylate + benzoate | 39 ± 1[c] | 3.5 ± 0.06[b] | 0.13 ± 0.03[b] |
| Glycine + benzoate | 116 ± 5[d] | 7.9 ± 0.40[c] | 0.90 ± 0.21[c] |

* Hepatocytes isolated from 18-hr fasted adult male rats were incubated for 1 hr at 37° in Krebs–Ringer phosphate solution supplemented with bovine serum albumin (1%), $NaHCO_3$ (25 mM), lactate (10 mM), $NH_4Cl$ (10 mM), and ornithine (5 mM). Values for urea and hippurate synthesis are averages ± SE (N = 3) in μmol/g liver (wet weight)/hr. Values for aspartate content are averages ± SE (N = 3) in μmol/g liver (wet weight) for hepatocytes isolated at the end of the incubation period. Values with different superscripts within a column are significantly different ($P < 0.05$).

zoate plus glyoxylate (2.0 mmol/kg) 1 hr prior to a challenge of ammonium acetate (9.0 mmol/kg), mortalities were 85, 30, and 57% respectively. At these doses, benzoate protected against ammonia toxicity, but glyoxylate compromised protection by benzoate. This observation prompted us to examine the metabolic effect of glyoxylate, alone and in combination with benzoate, on the urea cycle.

The possibility that glyoxylate, owing to its structural similarity to pyruvate, might inhibit ureagenesis by blocking aspartate regeneration via pyruvate carboxylase is supported by findings with isolated hepatocytes (Table 1). At a reaction concentration of 1 mM (well within the range for blood benzoate observed with clinical use [13]), glyoxylate and benzoate each inhibited the urea cycle about 50%, and the combination inhibited 74%. Consistent with the proposed site and mechanism, each drug depressed cellular aspartate levels about 80%. Although glycine and glyoxylate each stimulated conversion of benzoate to hippurate, only glycine restored ureagenesis and aspartate levels.

More direct evidence for the postulated site of action was obtained in assays with isolated mitochondria. Pyruvate-dependent $CO_2$ fixation by isolated mitochondria was inhibited sharply by glyoxylate and benzoate, with greater sensitivity to benzoate evident at concentrations below 1 mM (Fig. 1). Maximum inhibition by the combination of glyoxylate at 0.4 mM and benzoate over the range of concentrations shown was more severe than maximum inhibition by benzoate alone, suggesting different mechanisms of action.

It has been reported by others that the acid-stable radiolabeled product accumulated under these assay conditions is primarily a mixture of malate, fumarate, and citrate [14, 15]. This introduces ambiguity because oxaloacetate is not acid-stable and inhibition of citrate synthase by glyoxylate or benzoate could account for loss of acid-stable radiolabeled product. This problem of interpretation was resolved by modifying the assay to include glutamate and measuring only the fraction of $CO_2$ incorporated into aspartate by the coupled action of pyruvate carboxylase and glutamic oxaloacetic transaminase. While addition of glutamate did not alter the total amount of $CO_2$ fixed, it did shift the fraction incorporated into aspartate from 2 to 43% of that amount (Table 2, legend). A comparison of the effects of glyoxylate and benzoate, alone and in combination, on the incorporation of bicarbonate into total acid-stable product and into aspartate yielded virtually identical results (Table 2). The only known route for incorporation of radiolabeled carbon from bicarbonate into aspartate in isolated mitochondria is through the coupled action of pyruvate carboxylase and glutamic oxaloacetic transaminase. Radiolabeled carbon incorporated into citrate would not generate radiolabeled oxaloacetate (or aspartate) because the label would be lost as $CO_2$ during conversion of citrate to oxaloacetate by the reactions of the tricarboxylic acid cycle. Thus, inhibition at citrate synthase cannot account for the results obtained for pyruvate-dependent $CO_2$ fixation into aspartate.

That pyruvate carboxylase is a site of inhibition by both drugs is supported by measurements of pyruvate-dependent malate accumulation in isolated mitochondria, and pyruvate carboxylase activity in particle-free extracts. In the absence of added pyruvate, but under assay conditions otherwise identical to those given in Table 2, malate content in the acid-soluble fraction of the reaction mixture after a 10-min incubation was less than 1 nmol/mg mitochondrial protein. Addition of pyruvate sharply increased malate levels, to 86 ± 7 (mean ± SE; N = 3) nmol/mg protein. Consistent with preceding interpretations, glyoxylate and benzoate blocked the pyruvate-dependent rise in malate content by 94 and 79% respectively. Inhibition of pyruvate carboxylase by glyoxylate was confirmed in assays with particle-free extracts. A Lineweaver–Burk plot of data typical for assays of three preparations of mitochondrial extracts shows glyoxylate to be a competitive inhibitor of pyruvate binding to pyruvate carboxylase (Fig. 2). When oxaloacetate (100 μM) was substituted for pyruvate in this assay system, conversion to malate was >95% and insensitive to glyoxylate. On the basis of these data, we conclude that the reductions in $CO_2$ fixation and malate accumulation resulted from inhibition of pyruvate carboxylase.

Table 2. Pyruvate-dependent incorporation of [$^{14}$C]$KHCO_3$ into total acid-stable product and aspartate by isolated mitochondria*

| Additions or deletions | Incorporation of [$^{14}$C]$KHCO_3$ into Total acid-stable product (% Control) | Aspartate (% Control) |
|---|---|---|
| None (control) | 100[a] | 100[a] |
| −Glutamate | 99 ± 7[a] | 4 ± 1[b] |
| +Glyoxylate (1 mM) | 55 ± 2[b] | 50 ± 4[c] |
| +Benzoate (1 mM) | 30 ± 4[c] | 32 ± 7[d] |
| +Glyoxylate + benzoate | 15 ± 2[d] | 16 ± 5[e] |

* Assay conditions are described in the legend to Fig. 1 except that radioactive precursor was raised to 5–7.5 μCi and glutamate (3.5 mM) was added unless stated otherwise. Radiolabeled aspartate was isolated by cocrystallization with carrier. Total acid-stable radiolabeled product was determined as described in Materials and Methods. Values shown are averages for three or more preparations of mitochondria. The average values (nmol/min/mg protein ± SE; N = 7) for incorporation of bicarbonate were 23 ± 3 and 10 ± 1 for total acid-stable product and aspartate, respectively, with added glutamate (control), and 21 ± 2 and 0.4 ± 0.06, respectively, without glutamate (−glutamate). Values with different superscripts within a column are significantly different ($P < 0.05$).

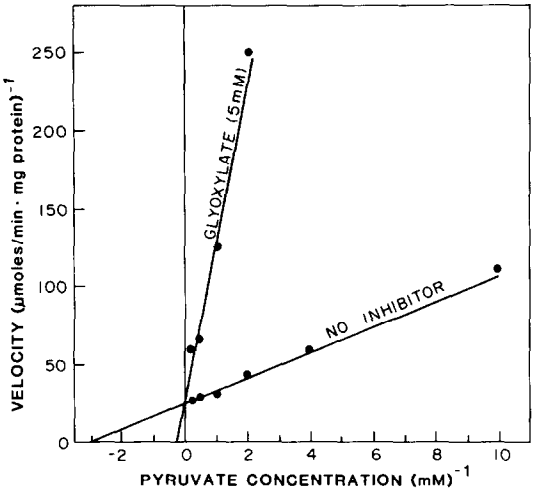


Fig. 2. Lineweaver–Burk plot of the effect of glyoxylate on pyruvate carboxylase kinetics. Particle-free sonicates of mitochondria in water were incubated at 30° for 4 min in 2-ml reaction mixtures containing acetyl CoA at 85 μM and sodium pyruvate at the concentrations shown (see Materials and Methods for details). Product formation was determined as pyruvate-dependent incorporation of [$^{14}$C]$KHCO_3$ into acid-stable product.

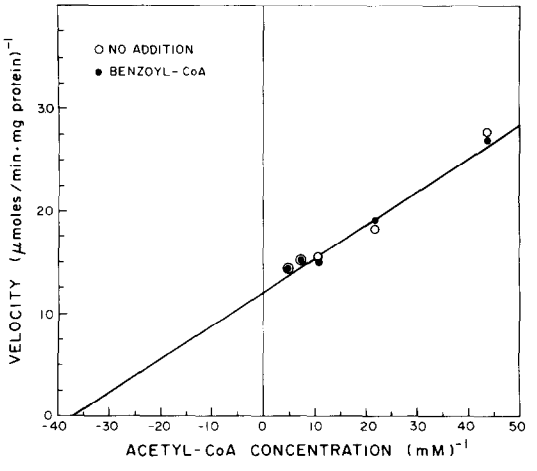


Fig. 3. Lineweaver–Burk plot of the effect of benzoyl CoA on the activation of pyruvate carboxylase by acetyl CoA. Conditions were the same as described in the legend to Fig. 2 except that the concentration of sodium pyruvate was 10 mM, benzoyl CoA 440 μM, and acetyl CoA as shown.

It is germane to note that products of glyoxylate metabolism may also inhibit pyruvate carboxylase. Oxalate is a potent non-competitive dead-end inhibitor [7] generated from glyoxylate in the peroxisomes [16]. In the assays with isolated mitochondria and mitochondrial extracts, kinetics obtained with glyoxylate indicate insignificant inhibition owing to oxalate generation, but oxalate formation could contribute to inhibition of pyruvate carboxylase upon longer exposure of hepatocytes to glyoxylate, as has been observed with other precursors [17]. Hydroxypyruvate, a product of transamination of glyoxylate with serine, also inhibited pyruvate-dependent $CO_2$ fixation by isolated mitochondria, but less effectively than glyoxylate; inhibition averaged 19 and 62% at 1 and 5 mM hydroxypyruvate respectively. Addition of aminooxyacetate (1 mM) blocked transamination by 92% (as indicated by measurements of the incorporation of [$^{14}$C]$KHCO_3$ into aspartate) but did not alter sensitivity of pyruvate-dependent $CO_2$ fixation to glyoxylate (data not shown). These results, and the absence of a lag phase for the action of glyoxylate on pyruvate-dependent $CO_2$ fixation, indicate that formation of hydroxypyruvate or other metabolites of glyoxylate are of minor importance to inhibition of $CO_2$ fixation by glyoxylate under our assay conditions. But inhibition by metabolites, particularly by oxalate, could well become significant *in vivo*.

Whereas glyoxylate is a competitive inhibitor of pyruvate carboxylase, inhibition by benzoate

appears to be a function of depletion of the activator, acetyl CoA, as a result of accumulation of benzoyl CoA [4, 5]. The alternative possibility that benzoyl CoA competes with acetyl CoA for the activator site was ruled out in assays with mitochondrial extracts. Benzoyl CoA at 440 $\mu$M did not influence activation by acetyl CoA over a range of concentrations from 23 to 200 $\mu$M (Fig. 3). Depletion of acetyl CoA introduces the possibility that benzoate may also interfere with ureagenesis at carbamoylphosphate synthetase, which is dependent on acetyl CoA for the generation of *N*-acetyl-L-glutamate, an essential activator of the enzyme [4]. Interference at this site is currently under study.

Results reported above demonstrate that benzoate and glyoxylate inhibit pyruvate carboxylase by different mechanisms, which might explain potentiation of benzoate toxicity by glyoxylate. Both drugs inhibited aspartate regeneration and urea synthesis in isolated hepatocytes, the combination more severely. These findings warn against combination therapy with benzoate and glyoxylate or its precursors without evidence of the efficacy of such therapy in a suitable animal model.

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