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THE EFFECT OF SODIUM BENZOATE ON AMMONIA TOXICITY IN RATS[1]

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SUMMARY: At 9.5 mmoles/kg body weight, sodium benzoate sharply increased mortality in rats subsequently challenged with ammonia. Fasted animals were less sensitive to potentiation of ammonia toxicity by benzoate than were fed animals. At 2.5 mmoles/kg body weight, benzoate was observed to protect fasted animals against ammonia toxicity. Measurements of ammonia disappearance, urea formation, and hippurate synthesis in suspensions of isolated hepatocytes indicate that benzoate potentiates ammonia toxicity by inhibiting the urea cycle.

INTRODUCTION: The administration of sodium benzoate to patients with genetic defects in the urea cycle has offered protection against ammonia toxicity (1,2). Protection is thought to result from utilization of ammonia to replenish the glycine spent in the elimination of benzoate as hippurate. Interest in extending benzoate therapy to combat ammonia toxicity in other disorders has been aroused by these clinical reports, but supportive evidence from laboratory tests has been elusive. Sodium benzoate sharply increased mortality in mice challenged with ammonia (3), failed to protect against hyperammonemia associated with portacaval shunting (4), and did not retard ammonia accumulation in suspensions of hepatocytes incubated with urease (5). Herein, potentiation of ammonia toxicity by benzoate is confirmed, and evidence on the mechanism is reported.

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MATERIALS AND METHODS: Male rats from the Charles River Colony (COBS-CD) weighing 200-250 g were used throughout. Upon receipt, rats were maintained on Purina Lab Chow ad libitum for 1-2 weeks before use. Ammonium acetate, sodium benzoate, and saline were administered ip in a total volume of 2 ml/200 g body weight. Hepatocytes were isolated from animals fasted 18 h as previously described (6) except that the perfusion medium was continuously gassed with O_2:CO_2 (95:5). Ureagenesis and hippurate synthesis were determined from measurements of the incorporation of [^{14}C]$NaHCO_3$ (10-15 uCi) and [1-^{14}C]benzoate (5 mM; 2-3 uCi) into urea and hippurate, respectively, by hepatocytes incubated for 1 h in 20 ml Krebs Ringer phosphate solution (7) supplemented with fatty-acid free bovine-serum albumin (1%) (Sigma Chemical Co), sodium bicarbonate (25 mM), sodium lactate (10 mM), ornithine (5 mM), and other additions as indicated. Radiolabeled products were isolated from the acid-soluble fraction by cocrystallization with carrier (5,6). Ammonia was determined from the oxidation of NADH catalyzed by glutamic dehydrogenase (EC 1.4.1.3) (8).

RESULTS AND DISCUSSION: Injection of ammonium acetate at 5.8 mmoles/kg to rats fed ad libitum yielded 10% mortality. Pretreatment with sodium benzoate at 9.5 mmoles/kg sharply increased mortality, from 10% to 90% (Table I, Series I), consistent with the report of O'Connor et al. (3). But mortality was not observed in the present study when benzoate was administered at the lower dose of 2.5 mmoles/kg.

At 9.5 mmoles/kg benzoate also potentiated ammonia toxicity in fasted animals; mortalities similar to those observed with fed animals required higher doses of ammonia (Series II). However, when the dose of benzoate was reduced to 2.5 mmoles/kg (a dose which is mid-range among those used in clinical trials) and the challenge of ammonia was increased to 7.7 mmoles/kg, protection against ammonia toxicity was observed; mortality was reduced from 63% to 13% (Series II).

It is well established that the availability of glycine limits the rate of hippurate synthesis (5,9,10). Although glycine is itself a ready source of ammonia, it reduced mortality from 90% to 60% when administered with benzoate prior to a challenge of ammonium acetate (compare Series I and III). This

TABLE I

THE EFFECT OF SODIUM BENZOATE ON MORTALITY IN RATS CHALLENGED WITH AMMONIUM ACETATE(A)

Pretreatment	Challenge (Ammonium Acetate)	Mortality expt 1	expt 2	Sum	%
Series I: FED ad libitum					
Saline	5.8	0/10	2/10	2/20	10
Benzoate, 9.5	5.8	8/10	10/10	18/20	90
Benzoate, 2.5	5.8	0/15	-	0/15	0
Series II: FASTED 18 hours					
Saline	5.8	1/10	0/10	1/20	5
Benzoate, 9.5	5.8	1/10	3/10	4/20	25
Saline	7.3	8/15	-	8/15	53
Benzoate, 9.5	7.3	15/15	-	15/15	100
Saline	7.7	11/15	8/15	19/30	63
Benzoate,2.5	7.7	0/15	4/15	4/30	13
Series III: FED ad libitum					
Benzoate, 9.5 plus Glycine, 9.5	5.8	9/15	9/15	18/30	60

(A) Male rats from the Charles River Colony (COBS-CD) weighing 200-250 g were maintained on Purine Lab Chow ad libitum for 1-2 weeks before use. Animals were pretreated 1 hour prior to the challenge of ammonium acetate. All substances were administered ip at the doses indicated in mmoles/kg body weight. Ammonia challenges were confirmed by assay with Nessler's Reagent.

suggests that benzoate potentiates ammonia toxicity through the accumulation of some intermediate in the conversion of benzoate to hippurate. Many substances metabolized through the formation of CoA esters interfere with some metabolic process in the liver (11). The accumulation of benzoyl CoA could interfere with ammonia detoxification via the urea cycle at two sites: (i) the intramitochondrial synthesis of carbamoylphosphate and (ii) the cytoplasmic condensation of citrulline with aspartate. Both reactions are indirectly dependent upon acetyl CoA, and the accumulation of benzoyl CoA could deplete acetyl CoA or inhibit its use. Acetyl CoA is a substrate in the synthesis of

TABLE II

THE EFFECT OF BENZOATE ON AMMONIA METABOLISM IN ISOLATED HEPATOCYTES[A]

Additions	Ammonia Disappearance (umoles)	Urea Formation (umoles)	Hippurate Synthesis (umoles)
NH_4Cl, 5 mM (100 umoles)	34.1	16.1	-
NH_4Cl, 5 mM plus Benzoate, 5 mM	9.5	8.0	0.4
NH_4Cl, 10 mM (200 umoles)	30.4	18.6	-
NH_4Cl, 10 mM plus Benzoate, 5 mM	14.4	9.7	0.3
NH_4Cl, 15 mM (300 umoles)	45.1	19.8	-
NH_4Cl, 15 mM plus Benzoate, 5 mM	20.1	12.0	0.3

[A] Hepatocytes equivalent to 185 mg liver wet weight were incubated under air for 1 h in 20 ml Krebs Ringer phosphate solution supplemented with bovine serum albumin (1%) sodium bicarbonate (25 mM), sodium lactate (10 mM) and ornithine, 5 mM; ammonium chloride and sodium benzoate were added as indicated. Incorporation of [^{14}C]$NaHCO_3$ (10-15 uCi) into urea was used to measure ureagenesis; ammonia content of the same reaction mixture was determined with glutamic dehydrogenase. Incorporation of [1-^{14}C]benzoate (2-3 uCi) into hippurate was measured in duplicate reaction mixtures. Radiolabeled products were isolated by cocrystallization with carrier (5,6). The same suspension of hepatocytes was used throughout. Replicate experiments gave similar results when corrected for the content of hepatocytes.

N-acetyl-L-glutamate, an essential activator of the ammonia-dependent carbamoylphosphate synthetase, and acetyl CoA is itself an activator of pyruvate carboxylase, an important source of aspartate (via oxalacetate) consumed in the urea cycle (12). Accordingly, the effect of benzoate on ureagenesis was examined in the following study with isolated hepatocytes.

At initial concentrations of ammonia ranging from 5 to 15 mM, benzoate inhibited the incorporation of [^{14}C] $NaHCO_3$ into urea in suspensions of hepatocytes by an average of 46% (Table II). Inhibition has been observed at concentrations of benzoate as low as 0.5 mM (data not shown). Reduction of ureagenesis by benzoate was not a result of diversion of ammonia from urea into hippurate; hippurate synthesis in duplicate reaction mixtures accounted for only 2% of the nitrogen which failed to appear in

urea as a result of the addition of benzoate (Table II). Indeed, more ammonia was available for ureagenesis in the presence of benzoate than in its absence; benzoate inhibited ammmonia removal by an average of 60% (Table II). The observation that glycine restored ureagenesis in the presence of benzoate to uninhibited rates (data not shown) rules out the additional possibility that inhibition of ureagenesis is a non-specific result of ATP depletion by benzoate metabolism. These results support the hypothesis that benzoate potentiates ammonia toxicity by retarding ammonia removal via the urea cycle. Such a mechanism urges caution with benzoate therapy for hyperammonemia in patients who benefit from a partially functional urea cycle, e.g. females heterozygous for ornithine transcarbamylase deficiency (13). Work is in progress to determine whether benzoyl CoA accumulates at toxic doses of the drug, and to identify the site(s) of inhibition of ureagenesis by benzoate.

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