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ON THE MECHANISM OF INHIBITION OF GLUCONEOGENESIS AND UREAGENESIS BY SODIUM BENZOATE

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Abstract—Synthesis of glucose from lactate and generation of urea from ammonia were inhibited when sodium benzoate was added to suspensions of rat hepatocytes. Assays with isolated mitochondria suggested pyruvate carboxylase and the *N*-acetyl-L-glutamate (NAG)-dependent carbamoylphosphate synthetase (CPS-I) as potential sites of inhibition for both pathways, owing to a shared dependency on aspartate efflux from the mitochondria and its subsequent conversion to oxaloacetate in the cytosol. Assays with isolated hepatocytes indicated inhibition to be initiated by accumulation of benzoyl CoA with a resultant depletion of free CoA and acetyl CoA. Measurements of adenine nucleotides showed that benzoate metabolism did not sufficiently alter energy status to account for the observed inhibition. Consistent with these interpretations, acceleration of the conversion of benzoyl CoA to hippurate by the addition of glycine restored the levels of free CoA and acetyl CoA and the rates of gluconeogenesis and ureagenesis. Reduction of the levels of aspartate and glutamate, presumably by interference with the anapleurotic function of pyruvate carboxylase, most likely accounted for inhibition of gluconeogenesis by benzoate. Whether reduced flux through the urea cycle also contributed to inhibition of gluconeogenesis (by diminishing cytosolic conversion of aspartate to oxaloacetate) requires further study. Depression of glutamate and acetyl CoA to levels at or below the K_m for NAG synthetase probably accounted for the observed inhibition of ureagenesis. Rates of urea production were observed to vary with changes in the levels of NAG, suggesting NAG-dependent CPS-I to be the primary site of inhibition of ureagenesis by benzoate.

Drugs detoxified through the intermediate formation of conjugates with coenzyme A can interfere with other CoA-dependent processes [1]. Accumulation of acyl CoA intermediates has been observed to inhibit gluconeogenesis [2–5], lipogenesis [2, 3], fatty acid oxidation [2–4, 6], pyrimidine biosynthesis [5, 7], and the urea cycle [3, 5, 7–9]. Sodium benzoate, a common food preservative detoxified via conversion to benzoyl CoA, has been employed successfully at fairly high doses (*ca.* 2 mmol/kg body weight) to combat ammonia accumulation in children with genetic defects in the urea cycle [10]. The rationale behind benzoate therapy is based on two considerations of its metabolism: (i) the substantial capacity of the body to eliminate benzoate as a glycine conjugate, hippurate, and (ii) the assumption that glycine spent in hippurate synthesis is replenished rapidly from endogenous pools of ammonia [10, 11]. Laboratory observations with mice genetically deficient in a urea cycle enzyme have been consistent with successful clinical results [12, 13], but several models of hyperammonemia in animals with a functional urea cycle have not been responsive to benzoate therapy [8, 14–18]. In some cases, benzoate unexpectedly potentiated ammonia toxicity, a contrary outcome apparently caused by interference with ammonia removal [8, 14, 18].‡

In examining the mechanism proposed for relief from ammonia toxicity, our attention was drawn to much earlier literature on laboratory animals and human subjects suggesting that glycine synthesis does not keep pace with its removal during benzoate metabolism [19, 20], a result consistent with our more recent finding that, with or without added ammonia, glycine availability sharply limits hippurate synthesis in suspensions of isolated hepatocytes [21]. Each of these observations has since been confirmed, in studies with rats [22, 23], mice [13], and human subjects [24, 25]. More detailed work with rats has shown the inadequate supply of glycine to cause accumulation of the intermediate, benzoyl CoA [2, 7, 23, 26].

We have suggested previously that accumulation of benzoyl CoA may result in inhibition of the urea cycle, and thereby account for potentiation of ammonia toxicity by benzoate [8]. Sequestration of CoA could retard ureagenesis by interfering with (i) energy metabolism [6], (ii) the role of pyruvate carboxylase in regeneration of aspartate [8], and (iii) ammonia-dependent generation of *N*-acetyl-L-glutamate (NAG) for activation of intramitochondrial carbamoylphosphate synthetase (CPS-I)

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‡ The term "ammonia" is used to designate the sum of NH_3 and NH_4^+ under the conditions specified.

[8], each of which is an acetyl CoA-dependent process.* In assays with isolated hepatocytes, described below, addition of benzoate was accompanied by accumulation of benzoyl CoA and depletion of acetyl CoA, but not by altered energy status. It is proposed that sequestration of CoA by benzoate leads sequentially to the observed reduction of flux through pyruvate carboxylase, depletion of aspartate, glutamate and NAG, and inhibition of gluconeogenesis and ureagenesis.

MATERIALS AND METHODS

Animals and materials

Male rats from the Charles River Colony (COBS-CD) weighing 200–300 g were used throughout. Rats were maintained on Purina Rat Chow No. 5012 *ad lib.* for 1–2 weeks after receipt, and fasted 18–21 hr before use. Radioisotopes and Aquasol (liquid scintillation fluid) were obtained from New England Nuclear, Boston, MA. Inorganic chemicals were purchased from Fisher Scientific, Springfield, NJ. All other chemicals and enzymes were obtained from the Sigma Chemical Co., St. Louis, MO.

Isolation of hepatocytes

Hepatocytes were isolated under ether anesthesia by the method of Seglen [27] according to details provided previously [5, 28]. Hepatocytes were washed twice by resuspension in 30 mL of Krebs–Ringer phosphate solution [29] containing $CaCl_2$ (1.25 mM) and finally diluted in this medium to yield 12–16 mg dry weight of cells/mL. The weight of the acid-insoluble lipid-free residue, measured according to Katz *et al.* [30], is reported below as the dry weight of cells. Viability, as assessed by exclusion of trypan blue, was >85%.

Incubation of hepatocytes for metabolite measurements

Hepatocytes equivalent to 60–80 mg dry weight of cells were incubated at 37° with swirling in sealed 50-mL Erlenmeyer flasks containing 20 mL of Krebs–Ringer phosphate solution [29], pH 7.4, supplemented with fatty acid-free bovine serum albumin (Fraction V, Sigma Chemical Co.) to a reaction concentration of 1% (w/v), $NaHCO_3$ (25 mM), lithium-lactate (10 mM), NH_4Cl (10 mM), ornithine-HCl (5 mM), $CaCl_2$ (1.25 mM) and other additions as indicated. Reactions were terminated as described for a given assay.

Metabolite assays

Values given for glucose, urea, orotate, and flux through pyruvate carboxylase were obtained from assays of the neutralized acid-soluble fraction of the complete reaction mixture. Values given for intracellular aspartate, glutamate, and NAG were obtained from neutralized acid-soluble (0.3 N $HClO_4$) extracts of cells harvested by centrifugation at 1000 *g* for 30 sec. The neutralized acid-soluble fraction of reaction mixtures or components thereof was routinely obtained by removing the acid-insoluble constituents through precipitation at 0.3 N $HClO_4$, followed by removal of perchlorate by precipitation upon neutralization with KOH. Values given for coenzyme A and derivatives and for adenine nucleotides were obtained from extracts of cells harvested through silicone oil, as described below.

Urea. Urea was assayed in a suitable dilution of the neutralized acid-soluble fraction of the incubation mixtures by the colorimetric method of Hunninghake and Grisolia [31]. Treatment of duplicate samples with urease prior to assay indicated >95% of the color formed was urea dependent.

Glucose. Glucose was determined in aliquots of the neutralized acid-soluble fraction by enzymatic coupling of glucose oxidase and peroxidase, using Sigma Diagnostic Kit No. 510. Glucose generated from endogenous sources was <5% of that generated from added gluconeogenic precursor.

Glutamate and aspartate. These metabolites were assayed by conventional enzymatic methods based on quantification of NADH [32]. Assays were verified by fortification of samples with authentic metabolite; greater than 85% of the metabolite added was accounted for, and benzoate did not interfere with the assays.

N-*Acetyl*-L-*glutamate.* The assay for NAG was based on the degree of activation of carbamoyl-phosphate synthetase in reaction mixtures containing lysed mitochondria from rat liver as the source of enzyme. The assay protocol requires a range of concentrations of authentic NAG to be assayed with each sample analyzed; the NAG content of the sample is obtained by graphic analysis of the results from each determination [33]. In our hands, cell extracts assayed before and after fortification with authentic NAG yielded 104 ± 4% (N = 4) of the value expected for the quantity of authentic NAG added.

CoASH and acylated CoA. Cell content of CoASH, acetyl CoA and benzoyl CoA was determined by adapting the procedure of Corkey *et al.* [34]. About 4 mg cells (dry wt) in 1 mL suspension of reaction mixture was centrifuged (13,600 *g* for 30 sec) through 300 μL of silicone oil (Dow Corning 550 and 220 oils in a ratio of 4:1, respectively) into 150 μL of 14% (w/v) $HClO_4$ containing dithiothreitol (2 mM) in an ice-cold microcentrifuge tube. The top layer and most of the oil layer were then aspirated off. An aliquot (125 μL) of the acid extract was transferred to an ice-cold 1.5-mL microcentrifuge tube containing 150 μL of a mixture of 1 M K_2HPO_4 and 2 M KOH (2.5:1) to adjust the pH to 4–5. The precipitated $KClO_4$ was removed by centrifugation, and 50 μL of supernatant fluid was analyzed immediately by HPLC as described previously [26]. Peaks were identified by comparison of retention time with that observed for authentic standards, and by co-chromatography after fortification of samples with authentic standards. Quantification was based on comparison of peak heights. Recovery of authentic CoASH, acetyl CoA and benzoyl CoA added to cell extracts was >90%.

Adenine nucleotides. Hepatocytes were separated

* During the course of this study, we learned that our working hypothesis was first proposed by S. J. Gatley (*Fed Proc* **36**: 672 (Abstr. 2114), 1977).

from the incubation mixture by centrifugation through silicone oil as described for analysis of CoA derivatives, except that dithiothreitol was omitted. The acid extract (125 μL) was diluted with water (285 μL) and 1 M K_2HPO_4:2 M KOH (2.5:1; 160 μL) to adjust the pH to 6.0–6.5. The diluted extract was then assayed for ATP and ADP by a modification of the ion-pair reverse phase HPLC method of Ingebretsen *et al.* [35]. Phosphate buffer (200 mM, pH 6.5) containing tetrabutylammonium hydrogen sulfate (0.9 mM) and methanol (5%, v/v) served as the mobile phase. Peaks were identified by retention times (11 and 14 min for ADP and ATP, respectively), co-chromatography after fortification of samples with authentic standards, and peak shift upon enzymatic transformation [35]. Quantification was achieved by comparison of peak heights to those obtained with known concentrations of authentic standards.

Incorporation of [^{14}C]$NaHCO_3$ *into orotic acid*

Cells were incubated as described above except that [^{14}C]$NaHCO_3$ (25–50 μCi) and 6-azauridine (10 mM) were added; 6-azauridine enhances orotate accumulation by blocking its conversion to UMP, but does not interfere with ureagenesis [28]. Radiolabeled orotate was isolated by cocrystallization with carrier [28].

Assay of pyruvate carboxylase in isolated mitochondria

Mitochondria were isolated and assayed for pyruvate carboxylase as described previously [26, 36]. Pyruvate-dependent incorporation of [^{14}C]$KHCO_3$ into acid-stable radiolabeled product was used as the measure of pyruvate carboxylase activity.

Assay of CPS-I in isolated mitochondria

Mitochondria (4–6 mg protein) were incubated for 10 min at 30° in 2-mL reaction mixtures consisting of KCl (37 mM), Tris (hydroxymethy) aminomethane (Tris)-HCl buffer (50 mM, pH 7.4), potassium phosphate buffer (5 mM, pH 7.4), potassium glutamate (10 mM), NH_4Cl (10 mM), ornithine-HCl (5 mM), and [^{14}C]$KHCO_3$ (25 mM, 0.5 to 1.0 μCi). Ammonia and ornithine-dependent incorporation of [^{14}C]$KHCO_3$ into acid-stable product was determined as described above for pyruvate carboxylase, and used as the measure of CPS-I activity.

Assay of NAG synthetase in solubilized mitochondria

Mitochondria (10 mg protein) were incubated for 15 min at 25° with shaking in 3-mL reaction mixtures of the following composition: Tris buffer, pH 8.5, 50 mM; arginine, 1 mM; glutamate, 10 mM; acetyl CoA, 0.175 mM; Triton X-100, 0.2%. Production of NAG was determined by measuring the difference in NAG content of the neutralized acid-soluble fraction at the start and end of the incubation period; NAG was assayed as described above.

Statistical analysis

Values are given as means ± SEM. Significance of difference between means given in Tables 1 and 2 was determined by Student's *t*-test for paired samples. Significance of differences shown in all other tables was determined by ANOVA followed by the Student–Newman–Keuls multiple comparison test. Least squares linear regression analysis was used to determine linear fit of data presented in Fig. 4.

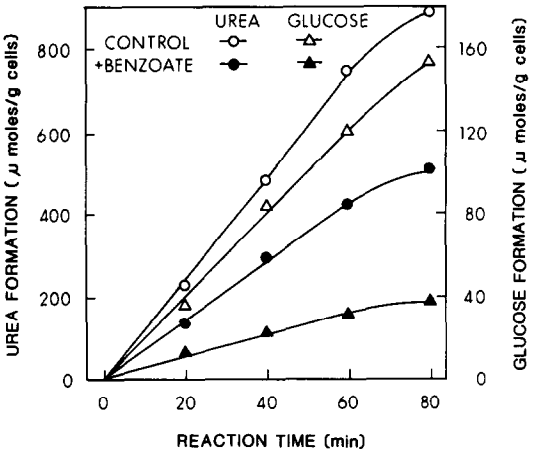

Fig. 1. Kinetics of urea and glucose formation in the presence and absence of benzoate. Hepatocytes isolated from male rats fasted 18–21 hr were incubated for the indicated times at 37° with lactate (10 mM), NH_4Cl (10 mM) and ornithine (5 mM). When added, benzoate was 1 mM. Urea and glucose were determined in the acid-soluble fraction of reaction mixtures. Results are averages from the assay of two preparations of hepatocytes.

RESULTS

Production of glucose and urea by hepatocytes incubated with lactate, ammonia and ornithine was linear for at least 60 min, whether or not sodium benzoate was included in the reaction mixture, and product formation was inhibited by sodium benzoate in both cases (Fig. 1).

At high ammonia, intramitochondrial carbamoylphosphate (CP) is generated more rapidly than it is consumed in citrulline synthesis, and the excess spills into the cytosolic compartment, where the expanded supply accelerates pyrimidine biosynthesis [37]. It has also been demonstrated that when hepatocytes are incubated with ammonia and lactate, pyruvate carboxylase serves as the major source of the carbon chain for generation of cytosolic aspartate ([38], Fig. 2). Accordingly, the nitrogen atoms of urea and the pyrimidine ring should be derived largely from the same pools of CP and aspartate under these conditions. This metabolic relationship was supported by results of a comparison of the sensitivity of the two pathways to increasing concentrations of benzoate (Fig. 3). Maximum inhibition of synthesis of urea and orotate was about 55% at 0.5 to 1 mM benzoate. Maximum inhibition of lactate-dependent gluconeogenesis was greater (85%) at lower concentrations (0.2 to 0.5 mM) of benzoate.

The preceding results are consistent with inhibition of pyruvate carboxylase (EC 6.4.1.1) and CPS-I (EC 6.3.4.16) through sequestration of coenzyme A, an interpretation supported by direct assay of each

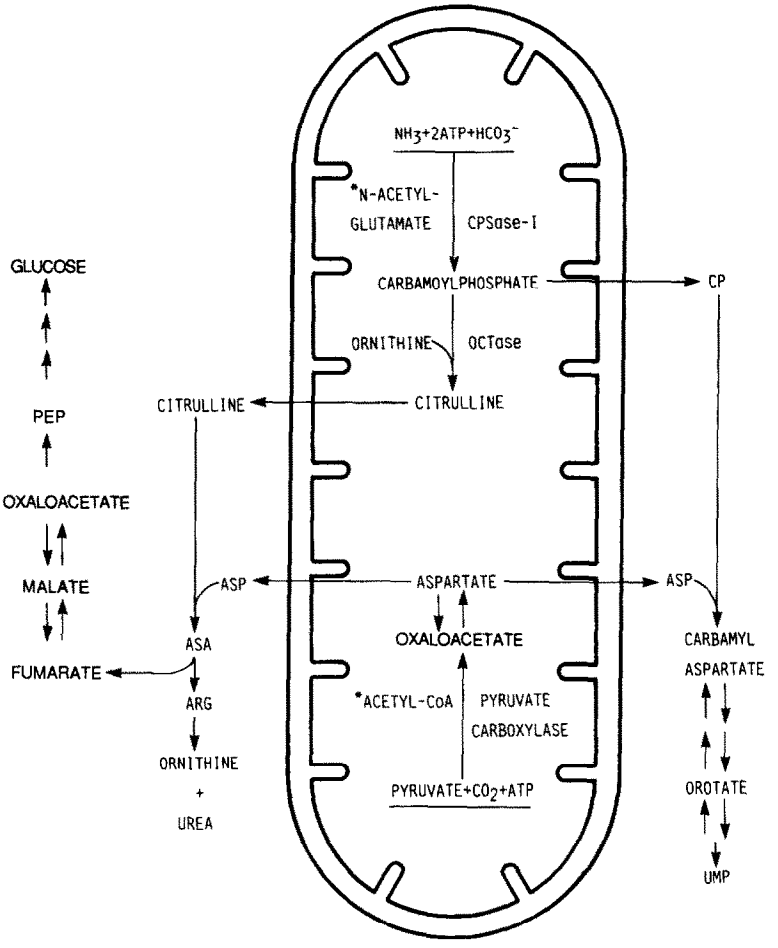

Fig. 2. Interrelationships between ureagenesis, lactate-dependent gluconeogenesis and pyrimidine biosynthesis at high ammonia. Asterisks identify proposed sites of inhibition by benzoate. Abbreviations: CPSase-I, carbamoylphosphate synthetase-I; OCTase, ornithine carbamoyltransferase; ASP, aspartate; ASA, argininosuccinate; ARG, arginine; PEP, phosphoenolpyruvate; and UMP, uridine monophosphate.

Table 1. Inhibition of carbamoylphosphate synthetase-I (CPS-I) and pyruvate carboxylase (PC) in isolated mitochondria by sodium benzoate*

	[^{14}C]$KHCO_3$ fixed (nmol/mg protein/min)	
Additions	CPS-I	PC
None (control)	11 ± 1	21 ± 2
Benzoate (1 mM)	8 ± 1†	7 ± 1†

* Mitochondria isolated from male rats fasted 18–21 hr were incubated for 10 min at 30° with glutamate (10 mM), NH_4Cl (10 mM) and ornithine (5 mM) in the assay for CPS-I, or pyruvate (5 mM) in the assay for PC. Values are means ± SEM for separate preparations of mitochondria (N = 16 for CPS-I; N = 10 for PC) and represent incorporation of [^{14}C]$KHCO_3$ (20–25 mM, 0.3 to 1.0 μCi) into acid-stable radiolabeled product.

† Significantly different ($P < 0.05$) from the corresponding control.

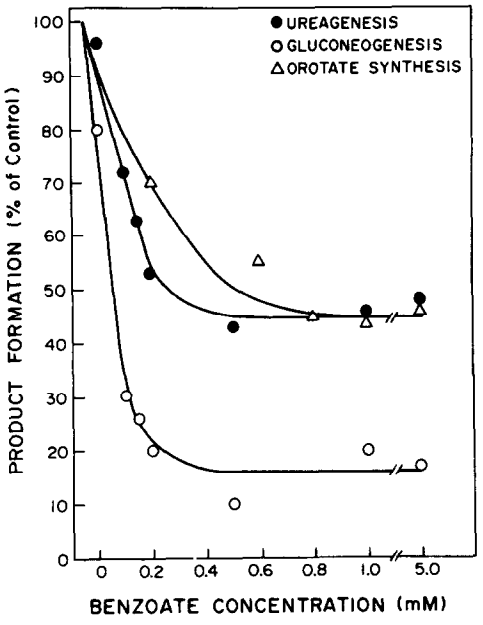

Fig. 3. Dose-dependent inhibition of ureagenesis, gluconeogenesis, and orotate synthesis by benzoate. Hepatocytes isolated from male rats fasted 18–21 hr were incubated for 30 min at 37° with lactate (10 mM), NH_4Cl (10 mM), ornithine (5 mM), and sodium benzoate at the concentrations indicated. Metabolites were determined in the acid-soluble fraction. Control rates (no benzoate added) for urea, glucose and orotate accumulation were 748, 104 and 2.52 μmol/g dry wt/hr. Results are averages from the assay of two preparations of hepatocytes.

enzyme in isolated mitochondria (Table 1). Both assays are based on incorporation of $[^{14}C]KHCO_3$ into acid-stable radiolabeled product. Results shown for CPS-I represent ammonia and ornithine-dependent incorporation (into citrulline) with glutamate as an energy source, whereas results for pyruvate carboxylase represent pyruvate-dependent incorporation (into malate, fumarate, and citrate [39]) with pyruvate as the energy source (ammonia and ornithine omitted). Product formation was linear for at least 12.5 min, with or without benzoate, and reduced more than 95% by omission of ammonia and ornithine or pyruvate (data not shown). Average inhibition at 1 mM benzoate was 27% for CPS-I and 67% for pyruvate carboxylase. Although inhibition of CPS-I was modest, it was statistically significant for both enzymes ($P < 0.05$).

Further evidence that inhibition of pyruvate carboxylase was responsible for reduced gluconeogenesis by isolated hepatocytes was obtained by substituting gluconeogenic precursors that bypass this enzyme. Dihydroxyacetone enters the gluconeogenic pathway at the intermediate stage of triose phosphates, and glutamine enters at the level of dicarboxylic intermediates in the citric acid cycle. Under conditions showing 68% inhibition of glucose production from lactate, glucose production from glutamine and dihydroxyacetone was not affected significantly by benzoate (Table 2). Since the ATP requirements to generate the quantities of glucose formed from dihydroxyacetone (2ATP/glucose) and lactate (6ATP/glucose) are comparable, interference with energy metabolism would not appear to explain the action of benzoate. These results point to pyruvate carboxylase as the primary site of inhibition of gluconeogenesis by benzoate, as suggested by earlier work [2].

If inhibition of gluconeogenesis and ureagenesis occurs as a result of sequestration of CoA, the inhibition should be relieved by providing glycine to regenerate free CoA (CoASH) through accelerated conversion of benzoyl CoA to hippurate [7, 21, 26]. The influence of benzoate and valproate, alone and in combination with glycine, on gluconeogenesis, ureagenesis, and flux through pyruvate carboxylase was examined in suspensions of the same hepatocytes. Valproate is known to inhibit pyruvate carboxylase by sequestration of CoA, but it is not metabolized to a glycine conjugate [3, 4, 26]. Flux through pyruvate carboxylase, calculated as 2Δglucose + Δglutamate + Δaspartate [40], consistently followed changes in gluconeogenesis and ureagenesis (Table 3). Addition of benzoate reduced flux by 89%, but addition of glycine with benzoate restored flux toward normal. In the same reaction mixtures, gluconeogenesis and ureagenesis were depressed 92 and 43% by benzoate, respectively, but to the lesser extent of 57 and 16%, respectively, when glycine was included. Similar findings have been reported for ammonia-dependent pyrimidine biosynthesis [7]. Valproate was a more potent inhibitor than benzoate, but inhibition by valproate was not modified by including glycine. Thus, relief by glycine was shown to correspond with its specific role as an acyl acceptor in benzoate metabolism.

The preceding interpretations were supported by assays of metabolite levels in hepatocytes harvested after incubation with and without benzoate. Hepatocytes that exhibited a 50% reduction in ureagenesis when incubated with benzoate showed no significant change in the level of ATP (5.0 ± 0.3 μmol/g dry wt) or in the ratio of ATP/ADP (4.7 ± 0.2; $N = 3$). However, the same hepatocytes exhibited a marked reduction in CoASH and acetyl CoA, 82% of which could be accounted for in accumulation of benzoyl CoA (Table 4). When glycine was included to antagonize the action of benzoate, the level of benzoyl CoA fell by 60%, CoASH and acetyl CoA rose almost 5 and 3 times, respectively, and ureagenesis was accelerated 1.9 times.

It has been reported that incubation of isolated rat hepatocytes for 30 min with lactate (2 mM), NH_4Cl (10 mM) and ornithine (3 mM) raises the hepatocyte content of NAG, glutamate, and aspartate 2.5, 3.7 and 37 times, respectively, and that the rise in NAG correlates well with acceleration of ureagenesis [41]. Several studies indicate that the rise in NAG in response to an ammonia load is mediated by elevation of glutamate levels [38, 42, 43]. Given its anapleurotic function, inhibition of pyruvate carboxylase might be expected to depress the level of glutamate as well as aspartate. Depletion of glutamate could interfere with the ability of the cell to generate NAG in response to an ammonia load. When this possibility was tested, incubation of

Table 2. Influence of sodium benzoate on gluconeogenesis from various precursors in isolated hepatocytes*

Gluconeogenic precursor	Glucose formation (μmol/g dry wt/hr)	
	Control	+ Benzoate (1 mM)
Lactate (10 mM)	75 ± 8	24 ± 4†
Glutamine (10 mM)	64 ± 9	54 ± 7
Dihydroxyacetone (10 mM)	235 ± 60	228 ± 56

* Hepatocytes isolated from male rats fasted 18–21 hr were incubated for 30 min at 37° with NH_4Cl (10 mM), ornithine (5 mM) and the gluconeogenic precursor indicated. Glucose was determined by enzymatic assay of the neutralized acid-soluble fraction. Values are means ± SEM for 3–5 preparations.

† Significantly different ($P < 0.05$) from the corresponding control.

Table 3. Inhibition of gluconeogenesis, ureagenesis, and flux through pyruvate carboxylase (PC) by benzoate and valproate*

Additions	Glucose formation (μmol/g cells/hr)	Urea formation (μmol/g cells/hr)	PC flux (μmol/g cells/hr)
None (control)	105 ± 20†	877 ± 36†	252 ± 38†
Benzoate (1 mM)	8 ± 5‡	503 ± 72‡	27 ± 15‡
Benzoate + glycine (5 mM)	45 ± 22§	740 ± 57§	119 ± 50§
Valproate (1 mM)	1 ± 0‡	366 ± 28‡	10 ± 2‡
Valproate + glycine	1 ± 0‡	363 ± 30‡	13 ± 3‡

* Hepatocytes isolated from male rats fasted 18–21 hr were incubated for 30 min at 37° with lactate (10 mM), NH_4Cl (10 mM), ornithine (5 mM) and other additions as indicated. All metabolites were measured in the acid-soluble fraction of each incubation mixture. Results are means ± SEM for three preparations of hepatocytes; PC flux was calculated as the sum of 2Δglucose + Δglutamate + Δaspartate.

†–§ Values with different symbols within a column are significantly different ($P < 0.05$).

Table 4. Effect of sodium benzoate on ureagenesis and the content of CoASH, acetyl CoA and benzoyl CoA in isolated hepatocytes*

Additions	Urea formation (μmol/g dry wt/hr)	CoASH	Acetyl CoA (nmol/g dry wt)	Benzoyl CoA
None (control)	678 ± 37†	396 ± 13†	177 ± 8†	ND
Glycine (5 mM)	689 (N = 2)	450 (N = 2)	183 (N = 2)	ND
Benzoate (1 mM)	340 ± 14‡	75 ± 4‡	39 ± 1‡	377 ± 23†
Benzoate + glycine	636 ± 39†	357 ± 24†	107 ± 11§	151 ± 8‡

* Hepatocytes isolated from male rats fasted 18–21 hr were incubated for 30 min at 37° with lactate (10 mM), NH_4Cl (10 mM), ornithine (5 mM) and other additions as indicated. Values are means ± SEM (N = 3) except where noted; ND = not detectable.

†–§ Values with different symbols within a column are significantly different ($P < 0.05$).

hepatocytes with lactate, NH_4Cl and ornithine under our assay conditions yielded at 2.4 times increase in NAG content within 30 min, and this rise in NAG was inhibited 72%, and urea production 42%, by the addition of benzoate (Table 5). Addition of glycine counteracted these inhibitory effects of benzoate. When the range of values observed for urea production was compared with the NAG content in hepatocytes harvested from these same reaction mixtures, a strong correlation was observed (Fig. 4). Declining production of glucose and urea was associated with parallel changes in the levels of aspartate, glutamate, and NAG (Fig. 5). On average, hepatocytes that exhibited a 70% decline in gluconeogenesis and a 38% decline in ureagenesis contained only 14% the level of aspartate and 27% the level of glutamate as did hepatocytes incubated without benzoate (Table 6). Again, inclusion of glycine with benzoate restored fluxes and metabolite levels toward control values.

Table 5. Effect of sodium benzoate on ureagenesis and *N*-acetyl-L-glutamate (NAG) content in isolated hepatocytes*

Additions	Urea formation (μmol/g dry wt/hr)	Cell content of NAG at t = 30 min (nmol/g dry wt)	Δ NAG (nmol/g dry wt)
None (control)	786 ± 69†	429 ± 28†	253
Benzoate (1 mM)	452 ± 48‡	247 ± 14‡	71
Benzoate + glycine (5 mM)	743 ± 58†	367 ± 25†	191

* Hepatocytes isolated from male rats fasted 18–21 hr were incubated for 30 min at 37° with lactate (10 mM), NH_4Cl (10 mM), ornithine (5 mM) and other additions as indicated. Values are means ± SEM (N = 4); at time zero, NAG content was 176 ± 20 nmol/g dry wt.

†, ‡ Values with different symbols within a column are significantly different ($P < 0.05$).

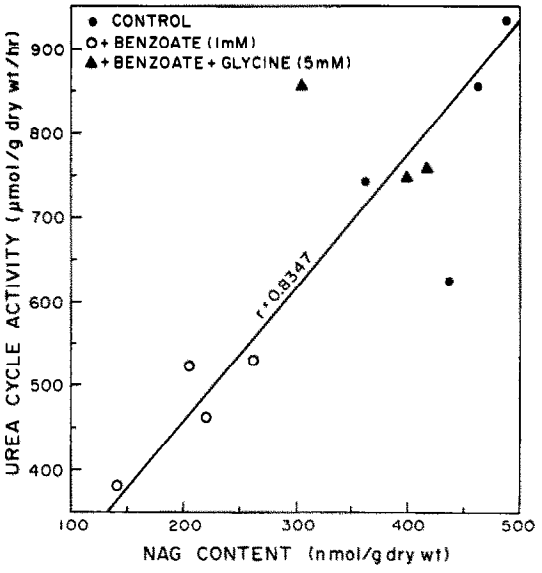

Fig. 4. Intracellular NAG content and urea cycle activity in isolated hepatocytes. By least squares linear regression analysis, $r = 0.8347$ and $P < 0.002$.

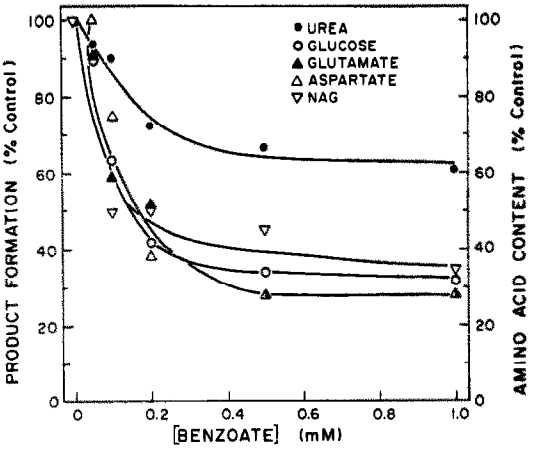

Fig. 5. Association of inhibition of ureagenesis and gluconeogenesis with a decline in the intracellular content of NAG, glutamate, and aspartate. Hepatocytes were incubated as described in the legend to Fig. 3. After 30 min, an aliquot of the reaction mixture was removed for assay of urea and glucose as described in the legend to Fig. 1, and cells were harvested from the remainder of the reaction mixture for assay of intracellular glutamate, aspartate and NAG. Control values were 618 and 186 μmol/g dry wt/hr for urea and glucose formation, respectively, and 8.5, 3.2 and 0.44 μmol/g dry wt for the cell content of glutamate, aspartate and NAG, respectively.

The possibilities that benzoate or benzoyl CoA interfered with the catalytic activity of NAG synthetase or blocked stimulation of CPS-I by NAG were eliminated in separate assays with solubilized mitochondria [44]. Synthesis of NAG occurred at a rate of 0.58 ± 0.08 nmol/mg protein/min (mean ± SEM; N = 6) and was unaffected by either benzoate (1 mM) or benzoyl CoA (0.57 to 0.72 mM; data not shown). Similarly, NAG-dependent activation of CPS-I was insensitive to either benzoate (1 mM) or benzoyl CoA (0.48 mM) over a range of NAG concentrations from 0.01 to 0.10 mM, which yielded a range of CPS-I activities from 0.7 to 5.0 nmol product/mg protein/min (data not shown).

DISCUSSION

In rat liver, the flow of carbon from lactate to glucose is dependent upon intramitochondrial CO_2 fixation by pyruvate carboxylase, followed by transamination and aspartate efflux [38]. At high ammonia, depletion of α-ketoglutarate in glutamate synthesis impedes regeneration of oxaloacetate from aspartate in the cytosol, and ammonia is thought to inhibit gluconeogenesis by thus depriving phosphoenolpyruvate carboxykinase of substrate [38]. High ammonia also accelerates the urea cycle, and the attendant cytosolic conversion of aspartate to fumarate may serve as an alternate route for directing the carbon chain of aspartate to oxaloacetate and glucose [38]. In this way, a block at either pyruvate carboxylase or CPS-I could conceivably inhibit production of both glucose and urea (Fig. 2). A block at either site could also inhibit pyrimidine

Table 6. Effect of sodium benzoate on ureagenesis and gluconeogenesis and the aspartate and glutamate content of isolated hepatocytes*

Additions	Formation of urea (μmol/g dry wt/hr)	Formation of glucose (μmol/g dry wt/hr)	Cell content of aspartate (μmol/g dry wt)	Cell content of glutamate (μmol/g dry wt)
None (control)	780 ± 51†	125 ± 9†	3.5 ± 0.4†	6.3 ± 0.7†
Benzoate (1 mM)	486 ± 17‡	37 ± 3‡	0.50 ± 0.05‡	1.7 ± 0.2‡
Glycine (5 mM)	683 (N = 2)	111 (N = 2)	2.3 (N = 2)	6.1 (N = 2)
Benzoate + glycine	748 ± 45†	98 ± 8†	1.3 ± 0.1§	3.7 ± 0.7§

* Hepatocytes isolated from male rats fasted 18–21 hr were incubated for 30 min at 37° with lactate (10 mM), NH_4Cl (10 mM), ornithine (5 mM) and other additions as indicated. At time zero, aspartate and glutamate contents were 0.4 ± 0.1 and 0.8 ± 0.1 μmol/g dry wet, respectively. Values are means ± SEM for 4–5 preparations of hepatocytes, except as noted.

†–§ Values with different symbols within a column are significantly different ($P < 0.05$).

Table 7. Compartmental concentration of substrates and activators compared to Michaelis and activation constants for key enzymes*

Enzyme (location)	Substrate or activator	K_a or K_m (mM)	Compartmental concentration (mM) observed in this study: − Benzoate	+ Benzoate
NAG synthetase (M)	Glutamate	3†	9.5	2.6
	Acetyl CoA	0.7†	0.5	0.1
CPS-I (M)	NAG	0.1–0.2†, 0.75‡	1.4	0.8
PC (M)	Acetyl CoA	0.378‡	0.5	0.1
ASA synthetase (C)	Aspartate	0.045†	1.5	0.2

* Symbols: (†) constants are literature values determined with enzyme purified from rat liver [40, 45–47]; (‡) value *in situ*, based on flux measurements with isolated hepatocytes [40]. Enzyme location: M (mitochondria); C (cytosol). Compartmental concentrations were calculated from values reported in Tables 4–6, assuming 83, 70, 35, and 44% of intracellular aspartate, glutamate, NAG, and acetyl CoA, respectively, are cytosolic in hepatocytes incubated with lactate, ammonia and ornithine [48, 49], and 2 mL cytosolic water and 0.2 mL mitochondrial matrix water per g dry wt of hepatocytes [49].

biosynthesis, which is dependent on flux through both enzymes at high ammonia.

Benzoate was observed to reduce production of glucose, urea, and pyrimidines when hepatocytes were incubated with lactate, ammonia, and ornithine. The reduction was attributed to inhibition of pyruvate carboxylase and CPS-I, owing to depletion of acetyl CoA through sequestration of CoA as benzoyl CoA (Tables 1 and 4). Calculation of compartmental distribution from established ratios for rat hepatocytes indicates that the intramitochondrial content of acetyl CoA in our studies fell from a value about 33% above the K_a to a value 1/4 the K_a for activation of pyruvate carboxylase, and from a value near the K_m to a value 1/7 the K_m for synthesis of NAG, the essential activator of CPS-I (Table 7).

Benzoate also sharply reduced the levels of aspartate and glutamate, as might be predicted from inhibition of flux through pyruvate carboxylase (Table 6). While glutamate levels fell from a value some 3 times above its K_m to a value slightly below its K_m for NAG synthetase, aspartate levels remained well above its K_m for argininosuccinic acid synthetase (Table 7). These results support the interpretation that depletion of acetyl CoA and glutamate each contributes to the failure of the cell to generate NAG for maximum activation of CPS-I in response to an ammonia load, but that reduced flux through pyruvate carboxylase appears sufficient to provide saturating levels of aspartate for conversion of citrulline to arginine. This finding is consistent with lack of evidence for citrulline accumulation; the colorimetric assay used for urea also detects citrulline, but insignificant levels of product were observed when samples were treated with urease before assay (data not shown).

A strong correlation was observed between changes in NAG content and urea production (Fig. 4), indicating reduced NAG levels were immediately responsible for impaired ureagenesis. However, reduced flux through pyruvate carboxylase likely plays a role in the inability of the cell to generate NAG, as evidenced by the parallel changes in NAG and glutamate levels (Fig. 5, Tables 5 and 6). Competition between acetyl CoA and benzoyl CoA for citrate synthase could also contribute to the short supply of glutamate [50].

Although benzoate was without significant effect

on ATP levels in isolated hepatocytes under our assay conditions, alterations in energy status may contribute to benzoate toxicity *in vivo*. Kalbag and Palekar [6] observed a sharp decline in hepatic ATP and a rise in hepatic ammonia 2 hr after administration of benzoate to rats, at about twice the typical clinical dose. Their evidence led them to conclude that the decline in ATP was not a result of uncoupling of oxidative phosphorylation, but due to ATP consumption for benzoate metabolism.

The results reported above support the conclusion that benzoate toxicity is triggered by depletion of acetyl CoA, owing to the accumulation of benzoyl CoA. The resultant inactivation of pyruvate carboxylase inhibits lactate-dependent gluconeogenesis and the supply of carbon for ammonia-dependent glutamate synthesis. Depressed levels of acetyl CoA and glutamate prevent accelerated rates of NAG synthesis in response to an ammonia load. The attenuated rise in NAG, an essential activator of CPS-I, reduces flux through the urea cycle. In judging the clinical relevance of our findings, it should be noted that the concentration of benzoate used in our studies is at the lower limit of the range of blood values reported for clinical trials [51, 52].

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