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EFFECT OF SODIUM BENZOATE ON CEREBRAL AND HEPATIC ENERGY METABOLITES IN spf MICE WITH CONGENITAL HYPERAMMONEMIA*

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Abstract—The sparse-fur (spf) mutant mouse has an X-linked deficiency of ornithine transcarbamylase and develops congenital hyperammonemia similar to that seen in human patients. We studied the effect of sodium benzoate (2.5, 5 and 10 mmol/kg body wt) on ammonia, glutamine and glutamate, as well as various intermediates of energy metabolism in brain and liver of normal CD-1/Y and hyperammonemic spf/Y mice. The ammonia concentration of brain was decreased with 2.5 mmol sodium benzoate in spf/Y mice, whereas higher doses resulted in a significant increase in both liver and brain. Cerebral glutamine content decreased generally in a dose-dependent manner, both in normal and affected mice, following treatment with various doses of sodium benzoate. Cerebral glutamate concentrations were increased only in spf mice treated with sodium benzoate, whereas ATP and acetyl CoA were decreased ($P < 0.001$), in both normal and affected mice, indicating that glutamine synthesis may be affected by ATP availability. Free CoA levels were decreased ($P < 0.05$) only in liver in both groups of treated mice, whereas pyruvate concentrations were elevated ($P < 0.05$) in affected mice following sodium benzoate administration. The results demonstrate that a dose of 2.5 mmol sodium benzoate/kg body wt has a beneficial effect in reducing cerebral ammonia with a concomitant decrease in glutamine. However, the results suggest that many of the metabolite changes observed following higher doses of benzoate could be due to depletion of ATP, free CoA and acetyl CoA levels, possibly secondary to benzoyl CoA accumulation. The response of the spf/Y mouse to sodium benzoate was different from that of the control CD-1/Y mouse, which could be due to its urea cycle dysfunction and a chronic hyperammonemic state. Hence, the spf/Y mouse may be the ideal animal model for studying the pharmacology of sodium benzoate in hyperammonemic disorders at both the cerebral and hepatic levels.

In 1979, Brusilow *et al.* [1] suggested that the conjugation of benzoate with glycine to form hippurate may be a significant alternative pathway for the excretion of waste nitrogen in children with hereditary urea cycle enzyme deficiencies. Batshaw and Brusilow [2], Msall *et al.* [3], and Brusilow and Horwich [4] further reported that the administration of sodium benzoate to various patients with genetic defects in the urea cycle offers protection against ammonia toxicity, improving the neurological outcome and survival time. Protection is thought to be the result of reduction of plasma concentrations of ammonia to replenish glycine, which conjugates with benzoate to form hippurate which is then eliminated in the urine. The glycine needed for the conjugation of benzoate is synthesized *de novo* by the liver, which is the principal limiting factor in hippurate synthesis [5, 6]. A recent report by Dass *et al.* [7] also showed that benzoate administered *in vivo* augments renal blood flow, glutamine extraction and total ammonium excretion by the kidney. This was supported by *in vitro* studies with separated rat renal proximal tubules, which demonstrated that benzoate stimulates glutamine-dependent ammoniagenesis by the activation of γ-glutamyl transferase (EC 2.3.2.2) via the synthesis of hippurate.

However, evidence from some other experimental studies has been less supportive of the use of benzoate as a therapeutic agent in hyperammonemia. O'Connor *et al.* [8] reported that benzoate, at a dose of 9.5 mmol/kg body wt, sharply increases the mortality in normal mice challenged with ammonium acetate. Benzoate at a concentration of 1 mM showed no effect on the accumulation of ammonia in normal hepatocyte suspensions when incubated with urease [6]. McCune *et al.* [9] observed an inhibition in glucose and fatty acid synthesis by normal rat hepatocytes following incubation with benzoic acid. They found that the addition of glycine prevented the inhibition of fatty acid synthesis caused by benzoic acid. Benzoate failed to protect against hyperammonemia associated with portacaval shunting in rats [10]. Maswoswe *et al.* [11], also reported an increased mortality with 9.5 mmol sodium benzoate/kg body wt in normal rats treated with ammonium acetate. They observed that this mortality rate was decreased when they injected glycine with

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benzoate prior to a challenge with ammonium acetate. These results suggest that benzoate may also potentiate ammonia toxicity by the accumulation of some intermediate during the conversion of benzoate to hippurate.

Benzoyl CoA, which is the intermediate for benzoate conjugation, has been shown to accumulate in both liver mitochondria [12] and hepatocytes isolated from normal rats [9] incubated with benzoate. These *in vitro* studies have shown that the addition of glycine diminishes the benzoyl CoA and increases hippurate synthesis by hepatocytes. Similar results have been shown in *in vivo* studies [13], in which rat liver glycine and serine concentrations decreased and benzoyl CoA accumulated subsequent to a treatment with 600 mg sodium benzoate/kg body wt. The accumulation of benzoyl CoA depletes the acetyl CoA or inhibits its use by competing with it. This could interfere with the detoxification of ammonia via the urea cycle [11, 14] by reducing the synthesis of *N*-acetyl glutamate, an allosteric cofactor for carbamyl phosphate synthetase-I.

The sequestration of CoA could also interfere with the hepatic energy metabolism by affecting the availability of CoA-SH, ATP and acetyl CoA. Gatley and Sherratt [12] observed an increase in total CoA content of isolated normal liver mitochondria when incubated with benzoate. McCune *et al.* [9] found significant decreases in free CoA and acetyl CoA levels in hepatocytes incubated with sodium benzoate. Kalbag and Palekar [15] reported a significant decrease in free CoA, ATP and acetyl CoA levels in normal rat liver homogenates following sodium benzoate treatment. Recently, Cyr *et al.* [14] also reported a significant decrease in free CoA levels of normal rat hepatocytes following incubation with benzoate.

It should be borne in mind that most of the above work regarding the therapeutic mechanism of benzoate was done using isolated hepatocytes from a normal animal with a functional urea cycle. Very little is known about its action on the detoxification of ammonia at the cerebral level. A widely proposed mechanism for ammonia toxicity is that excess ammonia interferes with brain energy metabolism [16]. Since the ammonia metabolism of brain and liver are intimately related, in both physiological and pathological situations, we considered it pertinent to study the effects of benzoate at both the cerebral and hepatic levels. In the present study, we have estimated the concentrations of ammonia, glutamine, glutamate and some of the intermediates of energy metabolism following sodium benzoate treatment.

As acute administration of ammonium salts to normal animals may not be an adequate model for human hyperammonemia, we chose to carry out these experiments in sparse-fur (spf) mice with the X-chromosomal defect of ornithine transcarbamylase (EC 2.1.3.3), which closely resembles the human inborn error of metabolism related to the urea cycle [17]. As it was also shown that benzoate affects the mitochondrial part of the urea cycle [11, 14], its response may vary in animals with inborn errors of the urea cycle when compared with normal animals. This could be the reason for the observed differences between the clinical and laboratory studies [14].

Our results showed a decrease in cerebral and hepatic ammonia levels with 2.5 mmol sodium benzoate/kg body wt, whereas higher doses led to increased ammonia levels both in brain and liver of spf mice. Glutamine content as well as ATP, free CoA and acetyl CoA levels were decreased significantly following higher doses of benzoate administration. The possible pathophysiological relevance of the findings along with the differences in the response of control and spf/Y mice to benzoate is discussed.

MATERIALS AND METHODS

Materials

ADP, ATP, α-ketoglutarate dehydrogenase, coenzyme A, acetyl coenzyme A and phosphotransacetylase were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium benzoate, lactic acid, pyruvate, glucose, α-ketoglutarate and glutamate were purchased from ICN Biochemicals, Cleveland, OH, U.S.A. NAD^+, NADH, NADP, lactate dehydrogenase, glutamate dehydrogenase, glucose-6-phosphate dehydrogenase, glutaminase and hexokinase were procured from Boehringer Mannheim, Mannheim, Germany. Blood ammonia test kits were obtained from the Technicon Instruments Corp., Tarrytown, NY, U.S.A. All other chemicals used were of analytical grade.

Animals

Sparse-fur male mice, of age 60–80 days, were used in the present study. At this age, the spf/Y mice are in an adaptive stage and are metabolically stable with a chronically hyperammonemic state [18]. The blood ammonia levels in this state are lower than those seen at the post-weaning stage. Sparse-fur mice, hemizygous for X-linked ornithine transcarbamylase (OTC) deficiency, were bred from the original Oak Ridge stock [17, 19]. These spf/Y animals were the progeny of matings of homozygous affected (spf/spf) females with normal (+/Y) males from a CD-1/Y background. All male progeny of these matings are affected with OTC deficiency. CD-1 strain male mice (Canadian Breeding Farms, St-Constant, Quebec) were used for the normal control studies. All mice were kept in a controlled environment (12 hr dark/12 hr light) with free access to water and food (Purina mouse chow). The mice weighed 35–40 g at the time of the experiment. The animals were kept and experimented upon according to the guidelines of the Canadian Council on Animal Care.

Experimental design

Determination of the time course of sodium benzoate treatment. Mice were injected i.p. with 2.5 mmol sodium benzoate/kg body wt and decapitated at 0, 30, 60 or 120 min after the injection. Five mice were used for each time course study.

Determination of the dose–response of sodium benzoate treatment. Mice were separated into four groups. One group (control) was given 0.15 M NaCl

solution (referred to in the figures as 0 mmol sodium benzoate/kg body wt); the other groups were given 2.5, 5 and 10 mmol sodium benzoate/kg body wt i.p. and were killed 1 hr after the injection. There were five mice in each group. No mortality was observed with any of the doses used in the present study.

Sampling methods. The mice were killed without anesthesia by decapitation. The brains were removed and frozen immediately (within 30 sec) in liquid nitrogen, following the method of O'Connor *et al.* [20]. The procedure of whole body immersion in liquid nitrogen before dissecting out the brain was neither considered necessary for this comparative study, nor feasible since blood and liver samples were also needed to verify the mutant status of the animals. Livers were excised and frozen in liquid nitrogen. Blood was collected from the neck wound and the serum was collected by centrifuging at 3500 rpm for 10 min. The brain and livers were weighed and immediately homogenized in 2 mL of 5% ice-cold perchloric acid using a "Polytron" (Brinkmann) homogenizer. The homogenates were centrifuged and the neutralized supernatants were used for the biochemical studies.

Biochemical methods

Ammonia was estimated by employing the commercial blood ammonia test kit, which uses an ion exchange method followed by colorimetric measurement of isolated ammonia nitrogen with the Berthelot phenate-hypochlorite reaction [21]. Glutamine was hydrolyzed by glutaminase and the resultant ammonia was estimated as above.

Estimations of α-ketoglutarate, glutamate, glucose, ATP, free CoA, acetyl CoA, lactate and pyruvate in both cerebral and hepatic samples were carried out on neutralized perchloric acid extracts using the assays as described by Williamson and Corkey [22]. α-Ketoglutarate in the sample was converted to glutamate in the presence of ammonium chloride, NADH and glutamate dehydrogenase. Using the same enzyme the glutamate in the sample was converted to α-ketoglutarate in the presence of NAD^+ and ADP. The ATP was converted to ADP with the concomitant phosphorylation of glucose to glucose-6-phosphate in the presence of hexokinase. The glucose-6-phosphate was converted to 6-phosphogluconate with glucose-6-phosphate dehydrogenase; in this process NADP is reduced to NADPH. Glucose was measured using the same principle. ADP in the sample was estimated by coupling to pyruvate kinase and lactate dehydrogenase. Free CoA levels were determined by using α-ketoglutarate dehydrogenase reaction followed by the arsenate-catalyzed phosphotransacetylase reaction to assay acetyl CoA in the same aliquot. Lactate was converted to pyruvate with the addition of lactate dehydrogenase in the presence of NAD^+. Similarly, pyruvate was converted to lactate by lactate dehydrogenase in the presence of NADH. In all the above assays the change in absorbancy of either NADH or NADPH at 340 nm was taken as a measure of the levels of metabolites. The results were expressed as micromoles per gram wet weight of tissue.

Cytosolic and mitochondrial ratios of NADH/NAD^+ were calculated according to the method of O'Connor *et al.* [20] by using the ratios of lactate/pyruvate and glutamate/α-ketoglutarate, respectively. Hepatic ornithine transcarbamylase activity was measured by using the method of Ceriotti [23] as modified for the liver tissue by Qureshi *et al.* [24] at pH 7.7 at 37°C. OTC activity was expressed as micromoles of citrulline formed per milligram of protein per hour.

Statistical analysis

The mean values were compared using the Newman–Keuls multiple range analysis test. A P value of 0.05 or lower was taken to indicate a significant difference from the corresponding controls.

RESULTS

Hepatic ornithine transcarbamylase activity

Hepatic OTC activity was found to be around 10% in hyperammonemic spf/Y mice (6.7 ± 0.8 μmol citrulline produced/mg protein/hr) compared to normal CD-1/Y mice (68.2 ± 4.0 μmol/mg protein/hr). Benzoate administration did not have any effect on the activity levels of this enzyme either in mutant or normal mice.

Serum ammonia and glutamine levels

The serum ammonia levels were increased 20–38% in normal CD-1/Y and spf/Y mice following treatment with 5 and 10 mmol sodium benzoate/kg body wt (Table 1). The glutamine levels were decreased up to 79% in a dose-dependent manner in spf/Y mice while there were no significant changes in normal CD-1/Y mice (Table 1).

Effect of sodium benzoate at different time intervals

Intraperitoneal administration of 2.5 mmol sodium benzoate resulted in a significant decrease of cerebral ammonia levels in spf/Y mice for up to 2 hr (Fig. 1). However, the magnitude of the decrease was reduced with time. There were no marked changes in hepatic ammonia content with this dose for even up to 2 hr. The brain glutamine content of spf/Y mice was decreased up to 60% with time following sodium benzoate injection with no alterations in the liver content (Fig. 1).

Both cerebral and hepatic ATP contents of spf/Y mice were decreased with time following an i.p. injection of sodium benzoate (Fig. 1). The glucose contents of both brain and liver were increased up to 40% in 2 hr following administration of 2.5 mmol sodium benzoate/kg body wt (Fig. 1). A significant decrease was observed in both cerebral free CoA and acetyl CoA levels 2 hr after the administration of 2.5 mmol sodium benzoate/kg body wt, whereas the hepatic free CoA (15%) and acetyl CoA (24%) levels were decreased with time up to 2 hr in spf/Y mice (Fig. 1).

There was an increase in the cerebral glutamate content with 2.5 mmol sodium benzoate treatment, while the hepatic levels remained unaltered (Table 2). Both cerebral and hepatic α-ketoglutarate contents were unaltered following benzoate treat-

Table 1. Concentrations of ammonia and glutamine in the serum of normal CD-1/Y and hyperammonemic spf/Y mice following treatment with sodium benzoate

Sodium benzoate (mmol/kg body wt)	Ammonia (nmol/mL)		Glutamine (nmol/mL)	
	CD-1/Y	spf/Y	CD-1/Y	spf/Y
0	51.2 ± 2.9	80.7 ± 8.6	47.0 ± 2.0	259.0 ± 23.0
2.5	61.0 ± 5.0	87.8 ± 5.7	45.0 ± 5.0	180.0 ± 5.7*
5.0	64.0 ± 2.5*	110.0 ± 9.0*	45.0 ± 3.0	93.5 ± 7.0*
10.0	74.4 ± 4.9*	112.0 ± 6.8*	42.5 ± 3.0	54.0 ± 3.7*

Animals were injected i.p. with sodium benzoate and were killed after 1 hr. Values are means ± SD of five different animals.

* Significantly different from 0 mmol/kg body wt ($P < 0.05$).

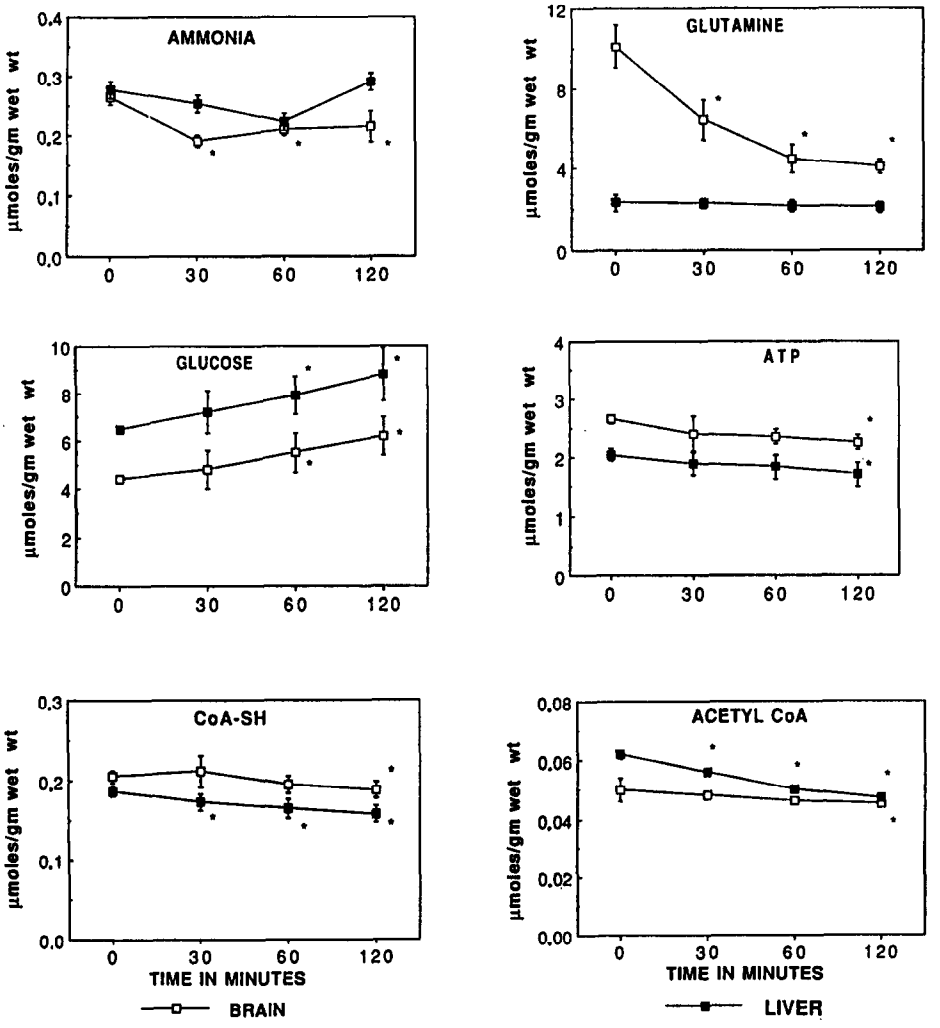

Fig. 1. Time-dependent effects of sodium benzoate on the levels of cerebral and hepatic metabolites of chronically hyperammonemic spf/Y mice. Mice were injected i.p. with 2.5 mmol/kg body wt and killed 0, 30, 60, or 120 min after the injection. Results are means ± SD from five different experiments. The values for glucose (liver) were multiplied by a factor of 0.1. Key: (*) $P < 0.05$ vs 0 time.

Table 2. Time-dependent effects of sodium benzoate on cerebral and hepatic metabolites in hyperammonemic spf/Y mice

Metabolite		Time after sodium benzoate injection (min)			
		0	30	60	120
Glutamate	C	5.72 ± 0.15	6.45 ± 0.35*	6.85 ± 0.45*	6.5 ± 0.4
	H	0.66 ± 0.06	0.60 ± 0.04	0.68 ± 0.07	0.7 ± 0.08
α- Ketoglutarate	C	0.31 ± 0.02	0.32 ± 0.02	0.32 ± 0.04	0.33 ± 0.05
	H	0.27 ± 0.02	0.28 ± 0.03	0.29 ± 0.03	0.30 ± 0.03
NADH/NAD$^+$	C	18.4 ± 0.5	20.2 ± 1.1	21.1 ± 1.4	19.7 ± 1.2
(mitochondria)	H	2.5 ± 0.2	2.2 ± 0.1	2.3 ± 0.2	2.4 ± 0.3
Pyruvate	C	0.16 ± 0.01	0.17 ± 0.10	0.17 ± 0.01	0.17 ± 0.1
	H	0.14 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.17 ± 0.01*
Lactate	C	7.5 ± 0.2	6.2 ± 0.8*	6.2 ± 0.4*	7.3 ± 0.9
	H	5.2 ± 0.1	5.5 ± 0.9	5.7 ± 0.3	5.8 ± 0.2*
NADH/NAD$^+$	C	46.9 ± 1.5	37.6 ± 4.8	36.9 ± 2.7	42.9 ± 5.3
(cytosol)	H	38.5 ± 0.9	37.2 ± 6.1	34.9 ± 2.1	34.1 ± 2.3

Values are means ± SD of five different animals. Sodium benzoate was administered i.p. at a dose of 2.5 mmol/kg body wt. C = cerebral levels; H = hepatic levels. Units: μmol/g wet wt of tissue, except for NADH/NAD$^+$.

* $P < 0.05$ vs 0-min time.

ment (Table 2). Cerebral pyruvate levels remained unaltered with the injection of sodium benzoate whereas the hepatic levels were increased with time (Table 2). Cerebral lactate levels were decreased following the administration of sodium benzoate for up to 1 hr and they approached the control level by 2 hr. Hepatic lactate levels were increased significantly 2 hr after sodium benzoate treatment (Table 2). Most of the above changes were more prominent at 1 hr and they remained more or less unaltered for up to 2 hr. We therefore decided to kill the mice after 1 hr for the dose-dependent studies. There were no significant changes in cytosolic and mitochondrial NADH/NAD$^+$ ratios following sodium benzoate treatment in either the brain or the liver (Table 2).

Effect of sodium benzoate at different doses

The cerebral ammonia levels of normal CD- 1/Y mice were unaltered with 2.5 and 5 mmol sodium benzoate but increased up to 21% with 10 mmol of sodium benzoate (Fig. 2). The hepatic ammonia levels of control mice were decreased up to 13% with sodium benzoate treatment but the changes were not statistically significant (Fig. 2). In spf/Y mice, both cerebral and hepatic ammonia levels were decreased 20% with 2.5 mmol sodium benzoate. However, both 5 and 10 mmol sodium benzoate/kg body wt resulted in an increase of ammonia levels in both brain and liver of spf/Y mice (Fig. 2). The cerebral levels of glutamine were decreased with all three doses of sodium benzoate in both normal and spf/Y mice (Fig. 2). The magnitude of changes in both ammonia and glutamine levels were greater in spf/Y mice than in the normal mice. The hepatic glutamine content was decreased to the same extent in both normal CD-1/Y mice and OTC-deficient spf/Y mice, with 10 mmol sodium benzoate/kg body wt; however, in spf/Y mice, it was not statistically significant (Fig. 2).

There were no significant changes in cerebral glutamate content of normal mice following treatment with benzoate, whereas in spf/Y mice it increased with all the three doses of benzoate (Table 3). Hepatic glutamate was increased with 10 mmol benzoate but there were no significant changes with lower doses of sodium benzoate in either group of mice (Table 4). Cerebral α-ketoglutarate levels were unchanged with benzoate treatment (Table 3), whereas the hepatic levels were increased in a dose-dependent manner in both groups of mice (Table 4).

A dose-dependent increase was observed in both cerebral and hepatic glucose levels of control and spf/Y mice with sodium benzoate (Fig. 2). Administration of 5 and 10 mmol sodium benzoate/kg body wt decreased the ATP levels markedly in both CD-1/Y and spf/Y mice (Fig. 3). The ADP levels were increased up to 22% in brain and 31% in liver with increasing doses of sodium benzoate (results not shown). There were no significant changes in the cerebral CoA levels in either normal or spf/Y mice except with 10 mmol sodium benzoate in spf/Y mice (Fig. 3). The hepatic CoA levels were decreased to a similar extent in both spf/Y and normal mice following the i.p injection of sodium benzoate (Fig. 3). A dose-dependent decrease was observed in acetyl CoA levels of both groups of mice with benzoate treatment (Fig. 3).

Pyruvate and lactate levels were unaltered in both cerebral and hepatic tissues of normal mice at any of the doses of sodium benzoate used (Tables 3 and 4). In spf/Y mice, the pyruvate levels were increased in both liver and brain with increasing doses of sodium benzoate (Tables 3 and 4). In the case of lactate, the cerebral levels were decreased with 2.5 mmol and remained unchanged with 5 mmol and increased significantly with 10 mmol benzoate/kg body wt (Table 3). Hepatic lactate levels increased steadily with increasing doses of benzoate in spf/Y

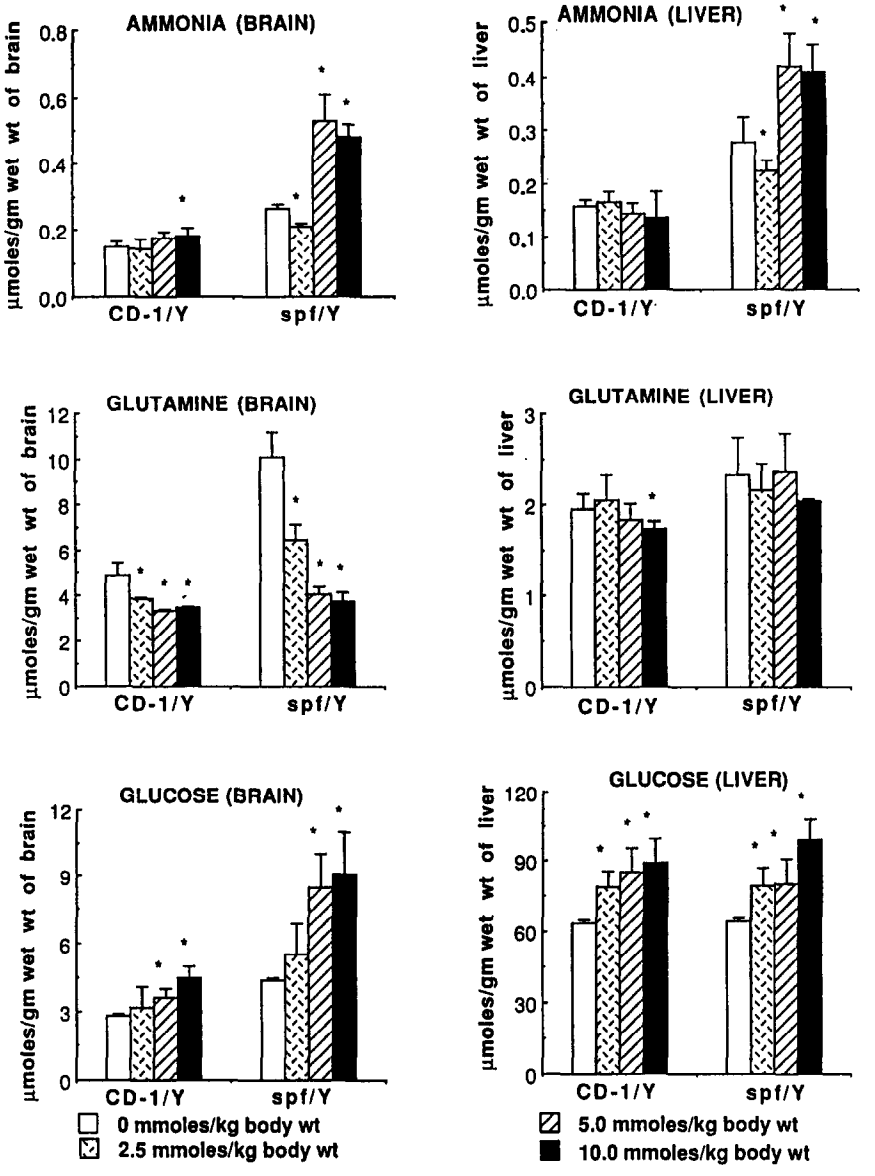

Fig. 2. Dose-dependent effects of sodium benzoate on the levels of ammonia, glutamine and glucose in normal CD-1/Y and chronically hyperammonemic spf/Y mice. Mice were killed 1 hr after the i.p. injection of sodium benzoate. Results are means ± SD from five different experiments. Key: (*) $P < 0.05$ vs 0 mmol sodium benzoate/kg body wt.

mice (Table 4). Both the cytosolic and mitochondrial redox states remained unaltered with any of the sodium benzoate doses used in the present study in both groups of mice (Tables 3 and 4).

DISCUSSION

Our recent studies have shown that ammonia and glutamine levels in both brain and liver are significantly higher in spf/Y mice compared to CD-1/Y mice under normal conditions [25]. In the present study, following administration of 2.5 mmol benzoate/kg body wt (which is within the normal therapeutic range used for humans), cerebral ammonia levels in spf/Y mice decreased, whereas at doses of 5 and 10 mmol/kg body wt the ammonia levels increased (Fig. 2). This biphasic effect of benzoate has been alluded to earlier [26]. It can be explained on the basis of inadequate availability of glycine for hippurate synthesis. The capacity of tissues to replenish glycine, spent in hippurate synthesis, is poor [13, 27]. However, in normal mice, the sodium benzoate treatment potentiated the ammonia levels in brain only at 10 mmol/kg body wt level, with no significant changes in the liver ammonia content (Fig. 2). The present findings are consistent with earlier reports [28] which showed that the i.p. administration of benzoate at 10 mmol/

Table 3. Dose-dependent effects of sodium benzoate on cerebral metabolite levels of normal CD-1/Y and hyperammonemic spf/Y mice

Metabolite		Sodium benzoate (mmol/kg body wt)			
		0	2.5	5.0	10.0
Glutamate	CD-1/Y	8.6 ± 0.56	8.7 ± 0.09	8.9 ± 0.9	9.2 ± 0.9
	spf/Y	5.7 ± 0.15	6.9 ± 0.45*	6.1 ± 0.25*	6.3 ± 0.07.*
α-Ketoglutarate	CD-1/Y	0.15 ± 0.02	0.15 ± 0.01	0.16 ± 0.02	0.16 ± 0.02
	spf/Y	0.31 ± 0.02	0.32 ± 0.04	0.35 ± 0.05	0.36 ± 0.06
NADH/NAD$^+$	CD-1/Y	59.3 ± 3.9	57.2 ± 0.6	57.0 ± 5.8	57.5 ± 5.6
(mitochondria)	spf/Y	18.4 ± 0.5	21.1 ± 1.4	17.4 ± 0.7	17.5 ± 0.2
Pyruvate	CD-1/Y	0.24 ± 0.01	0.25 ± 0.03	0.26 ± 0.02	0.26 ± 0.02
	spf/Y	0.16 ± 0.01	0.17 ± 0.01	0.20 ± 0.02*	0.21 ± 0.01*
Lactate	CD-1/Y	2.7 ± 0.06	2.8 ± 0.3	2.8 ± 0.3	2.7 ± 0.3
	spf/Y	7.5 ± 0.2	6.2 ± 0.4*	8.3 ± 0.9	8.7 ± 0.9*
NADH/NAD$^+$	CD-1/Y	11.3 ± 0.2	11.4 ± 1.2	10.9 ± 1.3	10.4 ± 1.1
(cytosol)	spf/Y	46.9 ± 1.5	36.9 ± 2.7	41.0 ± 4.5	40.8 ± 5.2

Values are means ± SD of five different animals. Mice were injected with sodium benzoate at various doses and were killed at the end of 1 hr. Units: μmol/g wet wt of tissue, except for NADH/NAD$^+$.
* $P < 0.05$ vs 0 mmol sodium benzoate/kg body wt.

Table 4. Dose-dependent effects of sodium benzoate on hepatic metabolites of normal CD-1/Y and hyperammonemic spf/Y mice

Metabolite		Sodium benzoate (mmol/kg body wt)			
		0	2.5	5.0	10.0
Glutamate	CD-1/Y	1.2 ± 0.08	1.1 ± 0.09	1.3 ± 0.10	1.3 ± 0.07
	spf/Y	0.66 ± 0.06	0.68 ± 0.07	0.75 ± 0.07	0.85 ± 0.07*
α-Ketoglutarate	CD-1/Y	0.15 ± 0.01	0.17 ± 0.01	0.18 ± 0.02*	0.22 ± 0.03*
	spf/Y	0.27 ± 0.02	0.29 ± 0.03	0.31 ± 0.02*	0.33 ± 0.01*
NADH/NAD$^+$	CD-1/Y	8.1 ± 0.5	6.7 ± 0.5	6.9 ± 0.5	6.3 ± 0.3
(mitochondria)	spf/Y	2.5 ± 0.2	2.3 ± 0.2	2.4 ± 0.2	2.6 ± 0.2
Pyruvate	CD-1/Y	0.29 ± 0.02	0.29 ± 0.03	0.31 ± 0.03	0.32 ± 0.04
	spf/Y	0.14 ± 0.01	0.16 ± 0.01	0.19 ± 0.01*	0.19 ± 0.01*
Lactate	CD-1/Y	3.96 ± 0.12	4.1 ± 0.34	3.95 ± 0.4	4.2 ± 0.5
	spf/Y	5.20 ± 0.12	5.7 ± 0.35*	6.50 ± 0.8*	6.8 ± 0.9*
NADH/NAD$^+$	CD-1/Y	13.9 ± 0.4	13.9 ± 1.1	12.7 ± 1.3	13.2 ± 1.6
(cytosol)	spf/Y	38.5 ± 0.9	34.9 ± 2.2	35.0 ± 4.3	35.4 ± 4.7

Values are means ± SD of five different animals. Mice were injected with sodium benzoate at various doses and were killed 1 hr after the i.p. injection. Units: μmol/g wet wt of tissue, except for NADH/NAD$^+$.
* $P < 0.05$ vs 0 mmol sodium benzoate/kg body wt.

kg body wt causes a significant elevation in plasma and liver ammonia in normal rats. It is well known that ammonia is detoxified in most tissues by the formation of glutamine and urea [29]. Both these processes require ATP. The conversion of benzoate to benzoyl CoA by benzoyl CoA ligase (EC 6.2.1.2) utilizes intramitochondrial ATP and CoA [12]. Thus, benzoate has the potential to disturb ATP and free CoA levels. It was also postulated that for every mole of benzoyl CoA formed from benzoate, there is a net utilization of 2 moles of ATP [12]. The present observation of reduced ATP levels agrees well with this possibility (Fig. 3). Altered levels of free CoA and acetyl CoA may also result in an interference with the normal operation of the tricarboxylic acid cycle by affecting the pyruvate and α-ketoglutarate dehydrogenases and citrate synthase, which would further aggravate the ATP depletion. The decreased glutamine content of brain observed in the present study (Fig. 2) could be the consequence of decreased ATP content following benzoate treatment. This is consistent with a diminished glutamine synthesis seen in the presence of increasing ammonia levels observed at higher doses of sodium benzoate. Decreased hepatic glutamine levels were reported previously [30] in normal rats following benzoate treatment. The observed increase in glutamate in brain in the present study (Tables 3 and 4) could be due to its decreased utilization for glutamine synthesis.

The decrease seen in free CoA (Fig. 3) could result in diminished activity of pyruvate

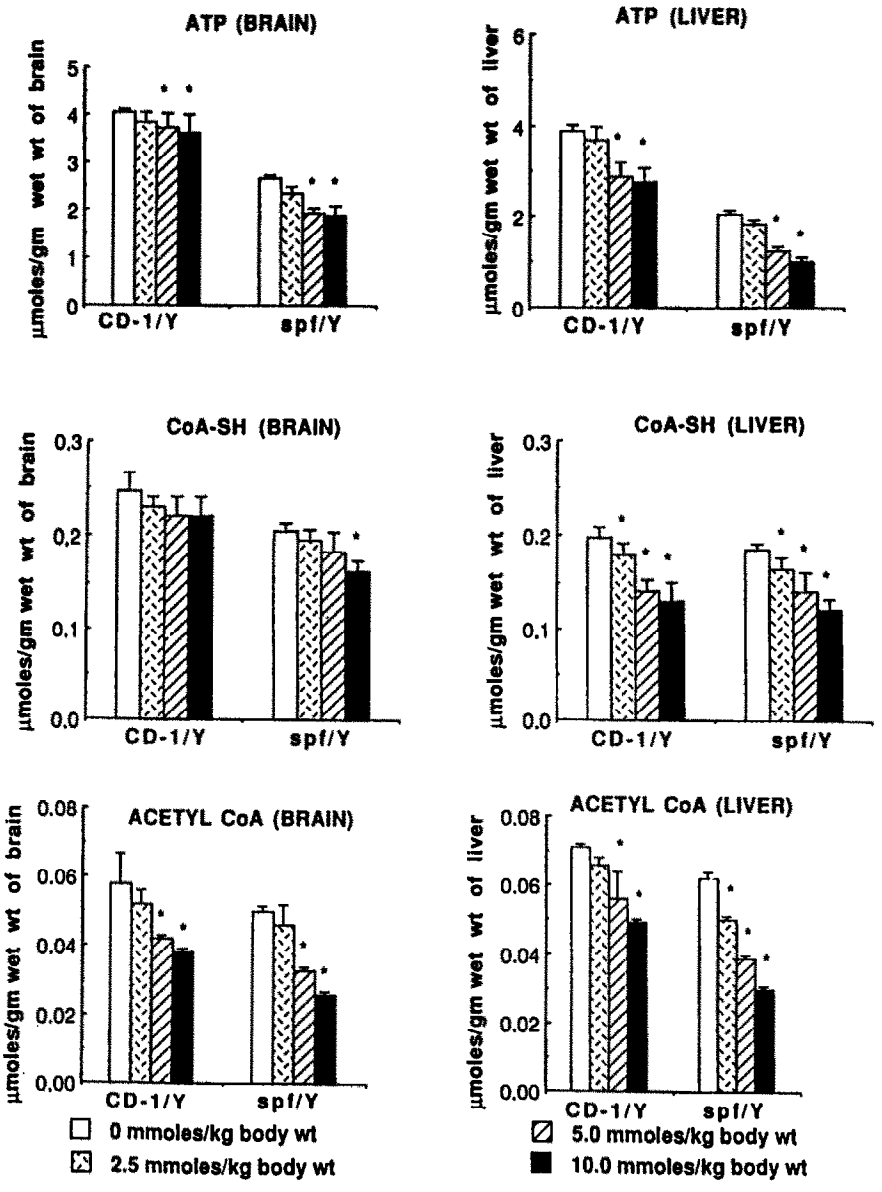

Fig. 3. Dose-dependent effects of sodium benzoate on the levels of ATP, CoA-SH and acetyl CoA in normal CD-I/Y and chronically hyperammonemic spf/Y mice. Mice were killed 1 hr after the i.p. injection of sodium benzoate. Results are means ± SD from five different experiments. Key: (*) $P < 0.05$ vs 0 mmol sodium benzoate/kg body wt.

dehydrogenase, offering a rational explanation for the increased levels of pyruvate and the decreased levels of acetyl CoA observed in the present study following benzoate treatment. Inhibition of pyruvate carboxylase by benzoate [14] might also explain the elevated pyruvate levels, and contribute to reduced flux through the citric acid cycle. The decreases in ATP, free CoA and acetyl CoA levels (Fig. 3) seen in the present study agree well with the results of Kalbag and Palekar [15], who reported similar changes in normal rat liver following sodium benzoate treatment. They also reported that sodium benzoate inhibits fatty acid oxidation in rat liver mitochondria. The observed decrease in acetyl CoA levels (Fig. 3) thus strengthens the likelihood that sodium benzoate is an inhibitor of fatty acid oxidation. Decreased ATP levels might also interfere with the normal activities of glycolytic enzymes like hexokinase and phosphofructokinase. This could probably explain the observed increase in the glucose levels in the present study (Fig. 2).

There were no significant changes in lactate levels in normal mice following benzoate treatment (Tables 3 and 4). However, 2.5 mmol sodium benzoate resulted in decreased cerebral lactate levels in spf/Y mice while 5 and 10 mmol doses led to increases,

which correlates well with the changes in the cerebral ammonia levels. Earlier reports [31, 32] on the enhanced levels of lactate in brain in various hyperammonemic states support the present observations on the changes of this metabolite with altered levels of ammonia. The reduced gluconeogenesis which occurs as a result of the inhibition of pyruvate carboxylase by sodium benzoate [14] could also result in elevated levels of lactate.

The magnitude of the changes observed by us in the intermediates of energy metabolism were more pronounced in liver than in brain, with the exception of glucose. As the liver is the major site for benzoate conjugation with glycine [12], it is possible that these changes in the levels of metabolites could take place primarily at the hepatic level. Since the liver is a primary source for metabolic fuels of the brain, some of these changes might also reflect in the brain, in addition to the changes taking place in the brain *in situ*. It has been reported that overall hippurate synthesis occurs at negligible levels in the rat and adult human brain [33]. It is not known, however, whether the lack of cerebral glycine conjugation is due to the physiological non-availability of the benzoyl CoA ligase or the benzoyl CoA-glycine transferase (EC 2.3.1.13). It could be possible that the benzoyl CoA ligase might be present in the brain, which will be mainly responsible for the observed depletion of ATP and free CoA levels.

It is evident from our studies that at low doses (2.5 mmol/kg body wt) sodium benzoate is effective in decreasing ammonia and glutamine, in both the brain and liver, without any significant changes in the levels of other metabolites and the cytosolic and mitochondrial redox states in the chronically hyperammonemic spf mouse. It was also reported that at the 2.5 mmol/kg body wt level benzoate protects fasted animals against ammonia toxicity [11]. The effect of sodium benzoate in causing a decrease in cerebral and serum glutamine levels in hyperammonemic spf/Y mice might point towards an important therapeutic role of benzoate in hyperammonemia. It has been shown recently that accumulation of glutamine may have a causative effect on the cerebral edema seen in hyperammonemic rats by serving as an ideogenic osmole [34]. Both serum and cerebral glutamine levels which were increased 60% and 98%, respectively, in spf/Y mice compared to control CD-1/Y mice were restored to the control levels following treatment with sodium benzoate. The other important observation of the present study was the increase in the cerebral glutamate seen in animals treated with sodium benzoate. It is well known that glutamate plays an important role as a excitatory neurotransmitter and also in the maintenance of the malate-aspartate shuttle. A fall in glutamate levels in hyperammonemic spf/Y mice compared to CD-1/Y mice may reduce the entry of reducing equivalents into the mitochondria. Sodium benzoate may ameliorate the situation by increasing glutamate levels.

The adverse effects of sodium benzoate, at higher doses of 5 and 10 mmol/kg body wt, at both cerebral and hepatic levels (depletion of coenzyme A and acetyl CoA) could be related to an accumulation of benzoyl CoA and a depletion or non-availability of metabolic glycine. As has already been shown earlier [9, 12], a supplementation with glycine could decrease benzoyl CoA and restore free CoA and acetyl CoA availability at the hepatic level. However, any use of supplemental glycine would be contraindicated in primary urea cycle disorders, as it would exacerbate hyperammonemia and contribute to ammonia toxicity at the cerebral level. Another compound which is known to enhance the pool of free CoA is carnitine, which has been shown to counteract the effects of benzoate at the level of hepatic mitochondrial *N*-acetylglutamate availability [35]. While some cerebral effects of L-carnitine administration in experimental models of hyperammonemia have been reported [36], no studies are known in which an interaction with benzoate therapy has been analyzed. It would, therefore, be interesting to see if L-carnitine, or its analogue acetyl L-carnitine, could reverse the adverse cerebral and hepatic effects of the higher doses of sodium benzoate seen in congenitally hyperammonemic spf mice. Acetyl L-carnitine has been shown recently to ameliorate the cerebral energy metabolism in experimental dogs [37].

In conclusion, sodium benzoate at a dose of 2.5 mmol/kg body wt could be effective in decreasing cerebral ammonia, glutamine and lactate, concomitantly increasing the glutamate levels in OTC-deficient spf/Y mice. These changes, except for glutamine, were not significant in control CD-1/Y mice. Hence, our studies indicate that the spf mouse, due to its urea cycle dysfunction and a chronic hyperammonemic state, may be the ideal animal model for studying the pharmacology of sodium benzoate at both the cerebral and hepatic levels.

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