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Failure of L-Carnitine to Protect Mice against Hyperammonemia Induced by Ammonium Acetate or Urease Injection

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ABSTRACT. Reports indicate that L-carnitine administration before 100% lethal dose of ammonium acetate suppresses the symptoms of ammonia toxicity and prevents death in mice. However, we have been unable to confirm this observation. The cause of discrepancy between our results and the results of others was investigated with two models of hyperammonemia in mice: *1*) that induced by intraperitoneal injection of urease and *2*) that induced by intraperitoneal injection of ammonium acetate. L-Carnitine administration failed to protect mice against ammonia toxicity induced by intraperitoneal injection of urease. Mortality in mice treated with L-carnitine 30 min before injection of ammonium acetate was similar to that of controls pretreated with saline. Ammonia and urea levels in plasma, liver, and brain were also similar in both groups. However, the values were significantly lower than those in mice denied either pretreatment before the ammonium acetate challenge. These results indicate that pretreatment acts to reduce blood and tissue ammonia simply by diminishing the rate of absorption of the challenge, owing to the dilution of ammonium acetate upon mixing with the contents of the peritoneal cavity. Thus, any protocol that does not compare results of a putative protective agent with those obtained with an equal volume of solvents or saline runs the risk of ascribing protective property to the agent when the protection may, in fact, have been afforded by the solvent. (*Pediatr Res* 28: 256–260, 1990)

Abbreviations

i.p., intraperitoneal(ly)

Hyperammonemia occurs in several pathologic conditions such as cirrhosis of the liver, inborn errors of the urea cycle, and other hereditary disorders such as organic acidemia and hyperlysinemia. Elevated ammonia is toxic to the brain and leads to convulsions, coma, and death. Several approaches have been recommended to combat hyperammonemia. These include limiting nitrogen intake, improving protein quality, supplying dietary metabolites such as arginine, and increasing nitrogen excretion in the form of derived conjugates (1, 2). It has been reported that L-carnitine administration to mice prevents the lethal effects of acute hyperammonemia induced by ammonium acetate injection (3, 4). These authors proposed that L-carnitine decreases the ammonia in blood and brain by inducing ureagenesis. Another group of investigators carried out similar studies using an identical protocol and also reported that L-carnitine protected mice against ammonia toxicity (5). However, when we attempted to repeat this work, we were unable to demonstrate the efficacy of L-carnitine in reducing hyperammonemia (6). Hearn *et al.* (7) recently reported that the protective effect of L-carnitine was short lived and was not consistently reproducible. They observed that in some experiments 100% of rats treated with L-carnitine survived an ammonium acetate challenge, whereas in other experiments more than 60% of the animals died after an identical treatment (7).

The first objective of our study was to investigate the cause for discrepancy between our results and the results of the other two groups of investigators. The results described here indicate that L-carnitine treatment does not protect mice against ammonia toxicity induced by an i.p. injection of ammonium acetate and that the protection reported by others is a function of experimental procedure and not carnitine treatment.

O'Connor *et al.* (3) also reported that L-carnitine treatment protected mice against hyperammonemia induced by urease injection. They claimed that although animals injected with L-carnitine plus urease had higher levels of plasma ammonia than those injected with urease alone, they did not exhibit the usual symptoms of hyperammonemia. Boehm *et al.* (8) also reported that L-carnitine protected rats against sustained hyperammonemia induced by urease injection. Again, rats receiving urease plus L-carnitine developed higher levels of ammonia in plasma and liver than animals receiving urease alone, but L-carnitine-treated rats remained relatively asymptomatic. The second objective of our study was to investigate the effect of L-carnitine treatment on the hyperammonemia induced by urease injection. Again, our results yielded no evidence that L-carnitine protects against hyperammonemia. The third objective of our study was to examine the effect of storage of blood containing active urease on blood ammonia levels.

MATERIALS AND METHODS

Materials. L-Carnitine (injectable form, pH 7.0) was purchased from Sigma-Tau Co. (Rome, Italy). Urease, glutamate dehydrogenase, diacetylmonoxime, perchloric acid, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals. Male Swiss albino mice, weighing 25–35 g, and adult male albino rats (Sprague-Dawley), weighing 250–300 g were purchased from Charles River Laboratories (Wilmington, DE) and were maintained on Rodent Chow (Ralston-Purina Co., St. Louis, MO). All animal experimentation was performed with Institutional Animal Care and Use Committee approval.

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Experimental procedure. In the first animal model, mice were divided into four groups according to treatment as follows: *1*, saline (injectable form, pH 7.0); *2*, ammonium acetate (pH 6.9); *3*, saline plus ammonium acetate; and *4*, L-carnitine plus ammonium acetate. Mice from groups 1 and 3 were injected (i.p.) with 0.25 mL of saline and mice from group 4 were injected (i.p.) with 16 mmol/kg of L-carnitine as a 20% (wt/vol) solution in water. Thirty min later, ammonium acetate (12 or 14 mmol/kg as 0.8 M solution) was injected (i.p.) into mice from groups 2, 3, and 4. In one experiment, mortalities were recorded (Table 1). In a second experiment mice were killed exactly 5 min after ammonium acetate injection (Table 2). Blood was collected from the heart in chilled, heparinized tubes. The livers and brains were quickly removed and immediately frozen. Ammonia and urea levels in plasma, liver, and brain were determined as described below.

In another set of experiments, the effect of L-carnitine on the sublethal dose of ammonium acetate was investigated (Table 3). Mice were injected i.p. with 16 mmol of L-carnitine/kg body wt. Thirty min later, they were injected i.p. with 9.0 mmol of ammonium acetate/kg body wt. Mice were killed exactly 15 or 60 min after ammonium acetate injection. Blood and brains were obtained as described above. Ammonia and urea levels in plasma and brain were determined as described below.

In the second animal model, mice were divided into two groups. Group 1 received a single injection of saline plus urease (i.p. 800 IU/kg) and group 2 received a single injection of L-carnitine (16 mmol/kg) plus urease (i.p., 800 IU/kg). Mortalities were recorded in one experiment (Table 1) and in a second experiment, the animals were killed 4 h after urease injection for determination of tissue ammonia and urea (Table 4). Blood, liver, and brains were removed and immediately deproteinized with chilled perchloric acid (1 M). Ammonia and urea were assayed as described below.

The effect of storage with active urease in blood on ammonia was determined as follows. Hyperammonemia was induced in rats by injecting (i.p., 400 U/kg) urease. Blood was collected by heart puncture in heparinized tubes 6 h after urease injection and deproteinized with chilled perchloric acid (1 M) after storage for various time intervals. The acid soluble fraction was obtained after centrifugation (8000 × *g*, 15 min at 0–2°C) and neutralized with 1 M sodium hydroxide. Ammonia was determined as described below.

Ammonia and urea assays. Blood samples were centrifuged at 7000 × *g* for 15 min at 0–2°C. Brain and liver samples were homogenized in chilled perchloric acid (1 M). The acid soluble fraction was obtained by centrifugation at 10 000 × *g* for 15 min at 0–2°C and neutralized to pH 7.0 with 1 N sodium hydroxide. Ammonia and urea levels were determined in the plasma and supernatants as described below.

Ammonia in the supernatants and plasma was assayed by glutamate dehydrogenase reaction (9). Ammonia-free water was used as a blank and also for preparing all reagents. The change in absorbance was determined before and after the addition of glutamate dehydrogenase. Urea in plasma and tissues was determined using diacetylmonoxime-antipyrene as described by Cerriotti (10).

Statistical analysis. Values are expressed as means ± SEM. Differences were analyzed by *t* test and considered statistically significant when $p < 0.05$.

Table 1. *Effect of L-carnitine on mortality in mice challenged with either ammonium acetate or urease**

Treatment	Number of animals		% Mortality
	Injected	Died	
Saline + ammonium acetate	15	11	73
Carnitine + ammonium acetate	15	12	80
Saline + urease	8	1	13
Carnitine + urease	8	3	38

* In the 1st set of experiments, mice were injected with either saline or L-carnitine (16 mmol/kg). Thirty min later, ammonium acetate was injected (i.p., 14 mmol/kg) and mice were observed for mortality. In the 2nd set of experiments, mice were injected with urease (i.p., 800 U/kg). One h later, either saline or L-carnitine was injected and mice were observed for mortality.

RESULTS

We carried out preliminary experiments to determine the lethal dose of ammonium acetate with and without prior injection of saline. At 12 mmol/kg ammonium acetate, six out of eight mice died. When the same dose was administered 30 min after an injection of saline, only one out of eight mice died. Other investigators have also reported reduced mortality rates in animals that were pretreated with saline before ammonium acetate challenge, suggesting that saline may have a protective effect against ammonia toxicity (5–7). Another group used L-carnitine instead of saline and concluded that the decrease in mortality rate was due to the protective effect of L-carnitine against ammonia toxicity (3, 4).

All mice receiving ammonium acetate showed usual symptoms of hyperammonemia. We found no statistically significant difference in mortality between mice treated with either saline (0.25 mL) or L-carnitine (16 mmol/kg) before ammonium acetate (14

Table 2. *Effects of saline or L-carnitine on ammonia and urea levels in plasma, liver, and brain of mice challenged with ammonium acetate**

Parameter	Saline	Ammonium acetate	Saline + ammonium acetate	L-Carnitine + ammonium acetate
Ammonia				
Plasma (μmol/L)	185 ± 14	6323 ± 416[a]	3577 ± 147[a, b]	3118 ± 461[a, b]
Liver (μmol/g)	2.4 ± 0.5	5.5 ± 1.0[a]	3.5 ± 0.3[a]	5.6 ± 0.9[a]
Brain (μmol/g)	1.4 ± 0.1	3.3 ± 0.2[a]	2.3 ± 0.6[a, b]	2.3 ± 0.2[a, b]
Urea				
Plasma (mmol/L)	6.0 ± 0.6	7.3 ± 0.4	7.7 ± 0.3	7.0 ± 0.4
Liver (μmol/g)	4.7 ± 0.4	7.0 ± 0.3[a]	7.9 ± 0.7[a]	5.4 ± 0.3[b, c]
Brain (μmol/g)	3.4 ± 0.2	4.2 ± 0.3	3.8 ± 0.3	3.7 ± 0.1

* Mice were injected i.p. with either saline or L-carnitine (16 mmol/kg). Thirty min later, ammonium acetate was injected (i.p., 12 mmol/kg) into all animals. Five min after ammonium acetate injection, blood, liver, and brain were removed. Results are means ± SEM. $n > 6$. *a*, *b*, and *c* superscripts indicate statistically significant difference ($p < 0.05$) when compared with saline, ammonium acetate, and saline + ammonium acetate groups, respectively.

Table 3. *Effects of saline or L-carnitine on ammonia and urea levels in plasma and brain of mice challenged with sublethal doses of ammonium acetate**

Parameter	Time (min)	Ammonium acetate	Saline + ammonium acetate	L-Carnitine + ammonium acetate
Plasma ammonia (μmol/L)	15	2913 ± 181	2296 ± 140	2599 ± 256
	60	ND	1107 ± 342	1284 ± 197
Brain ammonia (μmol/g)	15	4.6 ± 0.1	4.6 ± 0.5	4.1 ± 0.4
	60	ND	3.0 ± 0.4	3.6 ± 0.3
Plasma urea (mmol/L)	15	9.3 ± 1.5	9.4 ± 0.5	8.9 ± 0.6
	60	ND	10.4 ± 1.0	12.3 ± 0.9

* Mice were injected i.p. with either saline or L-carnitine (16 mmol/kg). Thirty min later, ammonium acetate was injected (i.p., 9 mmol/kg) into all animals. Fifteen or 60 min after ammonium acetate injection, blood and brain were removed. Results are means ± SEM. $n > 5$. ND, not determined.

Table 4. *Effects of L-carnitine on ammonia and urea levels in plasma, liver, and brain of mice challenged with urease injection**

Parameter	Saline + urease	L-Carnitine + urease
Ammonia		
Plasma (μmol/L)	1967 ± 468	1801 ± 252
Liver (μmol/g)	3.5 ± 0.64	2.9 ± 0.33
Brain (μmol/g)	2.9 ± 0.32	3.1 ± 0.42
Urea		
Plasma (mmol/L)	1.1 ± 0.20	2.0 ± 0.20[a]
Liver (μmol/g)	3.1 ± 0.18	4.3 ± 0.14[a]
Brain (μmol/g)	2.3 ± 0.17	2.9 ± 0.16[a]

* Mice were injected with urease (i.p., 800 U/kg). One h later, either saline or L-carnitine (16 mmol/kg) was injected. Four h after urease injection, mice were killed and blood, liver, and brain were removed. Results are means ± SEM. $n > 6$. A superscript *a* indicates statistically significant difference as compared with saline + urease group.

mmol/kg) injection (Table 1). The severity of sickness was similar in both groups.

Intraperitoneal injection of ammonium acetate (12 mmol/kg), either alone or 30 min after injection of saline or L-carnitine, caused a rapid increase in plasma, liver, and brain ammonia (Table 2). There were no significant differences in the plasma, liver, or brain ammonia levels between the groups pretreated with L-carnitine or saline. However, ammonia levels in plasma and brain of mice denied pretreatment before the challenge with ammonium acetate alone were significantly higher than those pretreated with saline or L-carnitine (Table 2).

O'Connor *et al.* (3, 4) reported that L-carnitine accelerated urea synthesis and thereby lowered blood and brain ammonia. In their study, the animals that were not pretreated with any solution died within a few minutes after ammonium acetate injection, whereas the animals pretreated with L-carnitine survived. In our study, animals receiving 12 mmol/kg ammonium acetate died within 8 to 12 min after ammonium acetate challenge. Therefore, we determined ammonia and urea levels 5 min after ammonium acetate challenge. We found that the urea levels in plasma and brain of mice injected with L-carnitine followed by ammonium acetate were not significantly different from those in controls injected with saline followed by ammonium acetate. On the contrary, urea levels in liver were decreased in the animals that were pretreated with L-carnitine as compared with those pretreated with saline (Table 2).

In another experiment, we investigated the effect of L-carnitine treatment on hyperammonemia induced by injecting a sublethal dose of ammonium acetate (Table 3). At 15 or 60 min after ammonium acetate challenge, there were no statistically significant differences in the plasma or brain ammonia levels between animals pretreated with either saline or L-carnitine (Table 3). Ammonia levels in plasma of animals that were denied any pretreatment remained higher than those given pretreatment, although the difference was not statistically significant. At 15 and 60 min after ammonium acetate challenge, plasma urea levels in L-carnitine-pretreated animals were comparable to those of saline-pretreated animals.

Two groups of investigators reported that although rats receiving urease plus L-carnitine developed higher levels of ammonia in plasma and liver than the animals receiving urease alone, rats treated with L-carnitine remained relatively asymptomatic (3, 8). However, in one of these studies, blood was not deproteinized immediately after collection (8). Under these conditions, urease present in the blood could continue to produce ammonia from urea. To test the effects of storage with active urease on blood ammonia levels, we conducted a preliminary experiment of a similar nature to that of Boehm *et al.* (8). The level of ammonia obtained in the blood of urease-treated animals depended on the time at which blood was deproteinized (Fig. 1). Ammonia levels in the samples increased significantly within 15 min of drawing blood. Values reported in Tables 1 and 3 were obtained from blood deproteinized immediately after drawing.

Mice injected with either saline plus urease or with L-carnitine plus urease became lethargic within 2 h of injection. Both groups exhibited the usual signs of hyperammonemia such as lethargy,

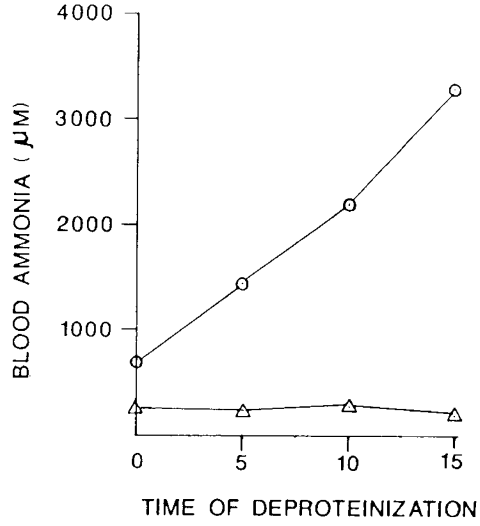

Fig. 1. Blood was collected 6 h after urease injection and deproteinized with 1 M perchloric acid at various time intervals. Ammonia was assayed as described in the text. △ = control (saline), ⊙ = experimental (urease).

convulsions, and coma. The severity of these signs was comparable in both groups. One out of eight mice from the saline-treated group and three out of eight from the carnitine-treated group died (Table 1).

The plasma, liver, and brain ammonia levels in mice administered L-carnitine plus urease were comparable to the levels observed with mice administered saline plus urease. Urea levels in the plasma, liver, and brains of mice treated with L-carnitine plus urease were significantly higher than those treated with saline plus urease (Table 4).

DISCUSSION

The major role of L-carnitine is to carry activated long-chain fatty acids across the inner mitochondrial membrane. L-Carnitine treatment has been reported to be effective against hyperammonemia induced by sodium valproate or propionic acid (11, 12). It has been suggested that L-carnitine may help to metabolize these acids by acting as a carrier or may make available intramitochondrial reduced CoA via acylcarnitine synthesis from CoA compounds already in the matrix (12). The mechanism by which L-carnitine protects against hyperammonemia has not been elucidated. According to O'Connor *et al.* (3, 4), L-carnitine treatment induces ureagenesis and thereby reduces hyperammonemia. However, we found that an injection of ammonium acetate caused a rapid increase in urea in liver, indicating that part of the ammonia may have been converted into urea via the urea cycle (6) (Table 2). We found that L-carnitine did not cause any additional increase in the urea levels in serum, brain, or liver above that obtained with ammonium acetate alone (Table 2).

It has been reported that i.p. administered L-carnitine accumulates preferentially in the brain (3, 4). Controversy exists regarding the ability of L-carnitine to cross the blood-brain barrier. According to an earlier report, the accumulation of L-carnitine by the brain from blood *in vivo* is slow (13), indicating that L-carnitine may not cross the blood-brain barrier readily. Hearn *et al.* (7) also reported that L-carnitine crosses the blood-brain barrier at a very slow rate. In the protocol used by O'Connor *et al.* (3), L-carnitine is administered only 30 min before ammonium acetate challenge. It is not clear whether the increase in L-carnitine content of brain observed in that study is sufficient to protect animals against ammonia toxicity. Studies carried out by Hearn *et al.* (7) indicate that the protection by L-carnitine against ammonia toxicity does not correlate with brain carnitine levels.

Injection of urease has been widely used to induce hyperammonemia in rats and mice. Our studies indicate that L-carnitine treatment was ineffective in protecting mice against ammonia toxicity induced by urease injection. The ammonia levels in plasma, liver, and brains of saline plus urease-treated animals were comparable to those of the L-carnitine plus ammonium acetate group (Table 4). These results are in contrast to those of O'Connor *et al.* (3), who obtained higher ammonia values in mice pretreated with L-carnitine than in those pretreated with saline. However, mice treated with L-carnitine plus urease had higher urea levels in plasma, liver, and brain than those treated with saline plus urease (Table 4). An i.p. injection of urease induces hyperammonemia by converting urea into ammonia, which is then reconverted into urea by the action of the urea cycle.

In another study, blood was not deproteinized immediately after collection (8). Under these conditions, urease present in the blood will continue to produce ammonia from urea. Therefore, blood ammonia levels obtained might be higher than the actual ammonia levels *in vivo*. Our results show that a misleading increase in ammonia concentration is obtained if the blood is not deproteinized immediately (Fig. 1). In the previous study (8), the animals may have been asymptomatic because ammonia levels *in vivo* were probably much lower than those reported.

Feeding an arginine-free diet to starved ferrets or cats also induced severe hyperammonemia within 3 h. Our earlier study has shown that L-carnitine treatment failed to protect ferrets against ammonia toxicity induced by an arginine-free diet (6). A recent report indicated that L-carnitine treatment also failed to reduce ammonia toxicity in postcaval-shunted rats (7). In this study, the ammonia levels in carnitine-treated animals were not different from those in untreated animals. However, there are conflicting reports about the role of L-carnitine protection against hyperammonemia, especially that induced by an i.p. injection of ammonium acetate. For example, two different groups of investigators claimed that L-carnitine treatment consistently protected mice (3, 5). Another group reported that in two experiments, the protection was significant but in the third experiment, L-carnitine did not protect rats against hyperammonemia (7). In our earlier as well as our present study, we were unable to reproduce the protective effect of L-carnitine (6). On the contrary, we observed that mice treated with L-carnitine plus ammonium acetate had higher ammonia in the liver than mice injected with saline plus ammonium acetate.

We were perplexed to note the differences between our results and those of other investigators who, at first impression, appeared to be using an identical protocol (3, 4, 6). However, a careful examination of these studies revealed a small but significant difference in the way control animals were treated. We injected (i.p.) control animals with a volume of saline equal to that of the putative protective agent administered before ammonium acetate challenge, whereas the other investigators did not pretreat their control animals with solvent or saline. In this report, we show that pretreatment with saline dilutes the effective dose or i.p. concentration of ammonium acetate, and this dilution renders the ammonium acetate challenge less toxic. Mice have a small peritoneal cavity and low blood volume. Inasmuch as all these experiments were carried out with mice weighing approximately 25 to 35 g, even a small difference in the volume of i.p. fluid could cause a significant difference in the molarity of a given dose of ammonium acetate in the peritoneal cavity. This may be the reason for failure of L-carnitine to suppress death in rats challenged with ammonium acetate (7, 14).

Our observation was further supported by the studies of Hearn *et al.* (7), who recently carried out similar experiments using two groups of controls. The protective effect of L-carnitine was observed only when the control group was denied a pretreatment with saline. This effect was completely abolished when controls were pretreated with an equal volume of saline. However, these authors speculated that the observed differences in the protective effect of L-carnitine could be due to differences in genetic or nutritional factors. Our results indicate that in all these studies control animals lacking pretreatment with saline were exposed to a higher i.p. concentration of ammonium acetate than experimental animals pretreated with carnitine and that this difference accounted for a higher survival rate in the experimental animals. This explains why a slightly higher concentration of ammonium acetate was required in our study to obtain a similar mortality rate (6) (Table 1). In our study, mice injected with L-carnitine and subsequently challenged with ammonium acetate succumbed as readily as mice injected with saline and ammonium acetate. Hearn *et al.* (7) reported that although 100% lethal dose for rats is 12 mmol/kg, many animals receiving this dose survived if they were pretreated with saline. This observation supports our hypothesis.

Earlier studies reported that L-carnitine treatment was more effective against hyperammonemia induced by injecting sublethal doses of ammonium acetate (7) and the maximum urea synthesis occurred at 1 h after ammonium acetate challenge (3). We tested the effects of L-carnitine on the hyperammonemia induced by 9 mmol/kg of ammonium acetate and observed that L-carnitine treatment did not reduce plasma or brain ammonia levels. There were no significant differences in the severity of sickness between the two groups. Compared with the control animals that were not challenged with ammonium acetate, mice

treated with L-carnitine plus ammonium acetate had increased urea levels at 15 and 60 min after ammonium acetate challenge (Tables 2 and 3). However, such an increase in urea was also observed in animals treated with saline plus ammonium acetate. Plasma urea levels in L-carnitine-treated animals were comparable to those in saline-treated animals (Table 3). These results confirm our earlier observation that L-carnitine treatment did not cause additional increase in urea above that obtained with ammonium acetate (6).

O'Connor *et al.* (4), in one of their studies, injected appropriate amounts of saline to control animals and L-carnitine to the experimental animals (4). It is difficult to evaluate this report because the results indicate that brain ammonia in carnitine-treated mice was twice as high as in saline-treated animals, whereas the authors concluded that L-carnitine caused reduction in brain ammonia. The values for brain ammonia in our studies (Table 2) for ammonium acetate or L-carnitine plus ammonium acetate groups suggest that L-carnitine treatment reduced ammonia levels in the brain. However, this protective effect of L-carnitine on brain ammonia disappears when the values are compared with those in the saline plus ammonium acetate-treated group.

To avoid discrepancies in results due to differences in animals or chemicals, we purchased the same strain of mice and obtained L-carnitine from the same source. One difference was that O'Connor *et al.* collected the blood from the tail vein, whereas we collected it from the heart. We were unable to collect sufficient blood from the tail veins of animals administered ammonium acetate, although sufficient blood could be drawn from the tail vein of the control animals. Another difference was that O'Connor *et al.* measured blood ammonia (blood was deproteinized and ammonia was assayed in supernatant), whereas we collected blood in heparinized tubes and measured plasma ammonia. These differences should not have any effect on our observation because the control and experimental animals were treated in an identical fashion and the same method for ammonia assay was used for all samples.

Symptoms of hyperammonemia have been reported to be delayed in several mice and in a few rats that were pretreated with L-carnitine (3, 7, 14). This was attributed to the protective effect of L-carnitine. However, we found delayed symptoms in mice treated with saline or L-carnitine plus ammonium acetate as compared with those treated with ammonium acetate alone. The symptoms of ammonia toxicity were probably delayed because of a decrease in the rate of ammonia absorption due to dilution by the solvent in the peritoneal cavity. The same alternative interpretation can be applied to the protection against ammonia toxicity by trimethylamine oxide, betaine, choline (5), t-butanol (15), and ethanol (16). We believe that if control animals are not injected with an equal volume of solvent or saline, any nontoxic substance will appear to protect against ammonia toxicity. Therefore, it is not surprising that even saline was reported as a protective agent against ammonia toxicity (5).

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