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CITRULLINE ACCUMULATION IN MICE INDUCED BY ADMINISTRATION OF L-HYDRAZINOSUCCINATE

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Abstract—L-Hydrazinosuccinate has been shown to induce a marked inhibition of liver aspartate aminotransferase isoenzymes in mice. The effects of the drug on the amino acid content of liver were studied. Intraperitoneal administration of L-hydrazinosuccinate enormously increased the citrulline content of liver and plasma in 6 hr and, less markedly, increased the glutamate and ammonia content of liver with a simultaneous decrease in the aspartate content. Drug administration also induced a marked increase in the liver mitochondrial activity of citrulline formation from ornithine, ammonia and carbon dioxide, with a similar increase in *N*-acetylglutamate content; a prominent increase in liver tryptophan dioxygenase activity; and an elevated level of plasma corticosterone. The increase of citrulline was interpreted to be produced by decreased conversion of citrulline to argininosuccinate due to a lack of aspartate because of inhibition of aspartate aminotransferase by the drug and increased formation of citrulline due to increases of glutamate and ammonia, which further induced the increase of *N*-acetylglutamate, because of inhibition of aminotransferase as well as stimulation of amino acid degradation by glucocorticoids.

L-Hydrazinosuccinate, the *N*-amino derivative of L-aspartate, has been shown to be a potent inhibitor *in vitro* of aspartate aminotransferase with a slow- and tight-binding manner of inhibition [1]. This inhibitor, administered to mice, was found to produce a potent inhibition of aspartate aminotransferase isoenzymes in liver and kidney [2].

Effects of these changes in enzyme activities on amino acid metabolism interested us and, therefore, we investigated the effects of L-hydrazinosuccinate administration on the amino acid content of liver.

The results included a remarkable accumulation of citrulline and are presented in this report.

MATERIALS AND METHODS

Treatment of animals with L-hydrazinosuccinate. Male DDY mice (25 g) kept on a standard diet (Oriental Yeast, Tokyo) were injected intraperitoneally with 0.6 mmol/kg body weight of L-hydrazinosuccinate acid neutralized with $NaHCO_3$ in 0.9% NaCl solution, while control animals received only saline.

After decapitation, blood was collected in beakers containing a small amount of heparin, and the plasma was obtained by centrifugation. Livers and kidneys were rapidly removed, washed in ice-cold saline, blotted dry, weighed and used for various purposes.

Amino acid analysis. Tissues were homogenized with 5 vol. of 5% trichloroacetic acid, and the homogenates were centrifuged at 10,000 *g* for 10 min. Portions (150 μl) of the supernatant fractions were diluted with equal volumes of 67 mM potassium citrate to adjust the pH to about 2.5 and were subjected to amino acid analysis. Plasma samples were treated with equal volumes of 8% trichloroacetic acid, and 250-μl portions of the extracts received an addition of 20 μl of 1 M potassium citrate for pH adjustment. Amino acids were analyzed, with a Beckman System 6300 High Performance Amino Acid Analyzer equipped with a cation-exchange resin column and connected to an SIC 7000 Integrator, by a programmed procedure of "Two Hour Physiological Fluid Analysis".

Colorimetric determination of citrulline was performed by the method of Boyde and Rahmaiullah [3]. Tissue extracts (20 μl) were treated with urease (0.4 units) for 15 min at 37° to remove urea before determination of citrulline.

Assay methods. N-Acetylglutamate in trichloroacetic acid extracts of liver prepared as described above was partially purified, before assay, by washing with ether and ion-exchange chromatography according to the method of Shigesada and Tatibana [4]. Determination of *N*-acetylglutamate in the samples thus prepared depended on its activation of carbamoyl phosphate synthetase as described by Zollner [5] and McGivan *et al.* [6]. The activation was measured by the formation of citrulline in a reaction mixture containing 20 mM ornithine, 20 mM NH_4Cl, 5 mM ATP, 15 mM $MgCl_2$, 10 mM $KHCO_3$, 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer, pH 7.2, in a total volume of 0.2 ml that also contained 0.1 ml of a sample solution and a supernatant fraction of sonicated rat liver mitochondria (protein, 0.6 mg). After 15 min of incubation at 37°, the reaction was stopped by the addition of 40 μl of 16% tri-

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chloroacetic acid, and the precipitates formed were removed by centrifugation. Citrulline content of the supernatant fraction was determined as described above. The sonicated extract of rat liver mitochondria was prepared by the method of McGivan *et al.* [7] with a mitochondrial preparation obtained by the method of Hogeboom [8].

Corticosterone was assayed by the fluorometric method of 11-hydroxycorticosteroids by Usui *et al.* [9]. Corticosterone was extracted into methylene chloride by vigorous shaking of plasma samples (0.2 ml) with the solvent (2 ml), and a 1-ml portion of the extract was mixed with 1.5 ml of a mixture of ethanol and sulfuric acid (3:7, v/v). Fluorescent emission at 520 nm was measured with excitation at 468 nm after 45 min.

Mouse liver mitochondria were prepared and assayed for citrulline formation activity as described by Yamazaki and Graetz [10], except that mannitol and Hepes were used in place of sucrose and Tris, respectively, and citrulline formation was not measured radiometrically but colorimetrically as described above.

Ornithine transcarbamoylase and carbamoyl phosphate synthetase activities were assayed as described by McGivan *et al.* [7]. Argininosuccinate synthetase activity was assayed according to the method of Ratner [11] except that citrulline was measured by the method of Boyde and Rahmaiullah [3]. Argininosuccinate lyase and tryptophan dioxygenase activities were assayed as described by Ratner [11] and Blake and Kun [12] respectively.

RESULTS

Effects of L-*hydrazinosuccinate treatment on hepatic levels of amino acids and ammonia.* Figure 1 shows the hepatic levels of some amino acids and ammonia in mice 6 hr after L-hydrazinosuccinate administration and compares them with the levels in the control mice. The inhibitor induced an enormous increase in the level of citrulline, which was negligible in the control. The increase of citrulline was confirmed by thin-layer chromatography of the liver extracts on Silica Gel 60 (Merck) with two solvent systems: (A) chloroform–methanol–17% ammonia (2:2:1, by vol.) and (B) ethanol–water (7:3, v/v). The figure also indicates that glutamate and a few other amino acids were increased. In addition, ammonia was increased to some extent. On the other hand, aspartate was found to be decreased markedly and alanine decreased to a lesser extent.

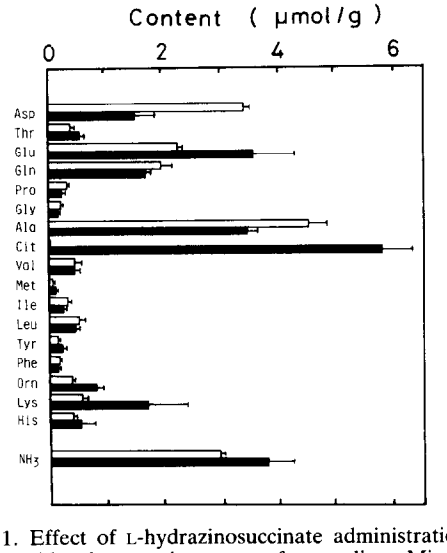

Fig. 1. Effect of L-hydrazinosuccinate administration on amino acid and ammonia content of mouse liver. Mice were injected with saline (white bars) or 0.6 mmol/kg of L-hydrazinosuccinate (black bars) and were killed after 6 hr. Trichloroacetic acid extracts of liver were prepared and analyzed as described in Materials and Methods. Values are means ± SD for three determinations, one from each animal.

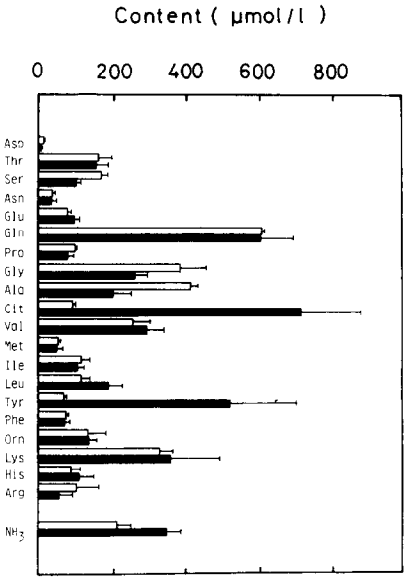

Fig. 2. Effect of L-hydrazinosuccinate administration on amino acid and ammonia content of mouse plasma. Mice were injected with saline (white bars) or 0.6 mmol/kg of L-hydrazinosuccinate (black bars). After 6 hr, plasma samples were obtained from the animals and analyzed as described in Materials and Methods. Values are means ± SD for three determinations, one from each animal.

As shown in Fig. 2, citrulline and ammonia in the plasma increased as in the liver after L-hydrazinosuccinate administration, whereas the level of glutamate appeared unaffected. A remarkable increase in the level of tyrosine was also observed. The plasma concentration of urea determined at the same time (not shown in the figure) was 6.00 ± 0.41 mM in the drug-treated animals, which is not significantly different from the concentration of 7.09 ± 0.87 mM in the control animals.

The time courses of the changes in the hepatic and renal citrulline levels are shown in Fig. 3. The citrulline content of liver increased rapidly after administration of L-hydrazinosuccinate, reached a maximum at about 6 hr, and decreased thereafter to a level slightly higher than the original level in 24 hr. The amino acid content of kidney followed a similar pattern of time dependence, but the change was very small.

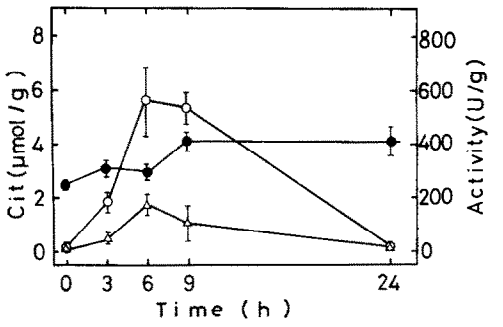

Fig. 3. Time course of the effect of L-hydrazinosuccinate administration on citrulline content of liver and kidney, and on liver argininosuccinate synthetase activity. Control mice were killed immediately after injection of saline, while the other mice were injected with L-hydrazinosuccinate (0.6 mmol/kg) and were killed at indicated times. Trichloroacetic acid extracts of liver (○) and kidney (△) were analyzed for citrulline as described in Materials and Methods, and crude cytosolic preparations of liver prepared as reported [1] were assayed for argininosuccinate synthetase activity (●). Values are means ± SD for three determinations, one from each animal.

Changes in enzyme activities related to the production and utilization of citrulline. To test the possibility that L-hydrazinosuccinate administration inhibits argininosuccinate synthetase *in vivo* and thereby causes the accumulation of citrulline, liver extracts obtained at various times after drug administration were assayed for argininosuccinate synthetase. As shown in Fig. 3, the activity did not decrease after injection of the inhibitor but, rather, increased slowly in contrast to the potent and long-lasting inhibition of aspartate aminotransferase previously reported [2]. The results indicate that the inhibitor did not produce any inhibition *in vivo* which was strong and persistent enough to be detected in the assay system *in vitro*, but the possibility still remains that argininosuccinate synthetase was inhibited significantly when it was being exposed to rather high concentrations of inhibitor *in vivo*. The possibility was tested by examining the *in vitro* effect of hydrazinosuccinate added to the assay system of the enzyme. There was no appreciable inhibition observed even with the inhibitor at a concentration of 5 mM, which was much higher than the average *in vivo* concentration of about 1 mM after the injection, suggesting that the possibility was very slight. In addition, the presence of L-hydrazinosuccinate in the assay system of argininosuccinate lyase was found to produce no significant inhibition of the enzyme when the experiments were conducted with a partially purified preparation of bovine enzyme (Sigma). Thus, the citrulline accumulation was hardly due to decreased activities of these enzymes in utilizing the amino acid, although the decreased level of aspartate described above may have slowed down the formation of argininosuccinate.

Table 1. Effect of L-hydrazinosuccinate administration on the rate of citrulline formation in mouse liver mitochondria*

Treatment of animals	Citrulline formation [nmol·min^{-1}·(g liver)$^{-1}$]
Control	681 ± 174
L-Hydrazinosuccinate	1622 ± 309

* Control mice were injected with saline and immediately killed; the other mice were injected with L-hydrazinosuccinate (0.6 mmol/kg) and were killed after 6 hr. Citrulline formation in liver mitochondria was assayed as described in Materials and Methods. Values are means ± SD for three determinations, one from each animal.

Attention was turned to the possibility of increased formation of citrulline. Formation of the amino acid in mitochondria isolated from L-hydrazinosuccinate-treated mice was compared with that from control mice. The data in Table 1 clearly indicate that citrulline formation was stimulated markedly in the mice treated with the inhibitor. To determine the reaction step(s) stimulated by the treatment, carbamoyl phosphate synthetase and ornithine transcarbamoylase activities were examined, but no significant difference was observed between the treated and control animals with respect to enzyme activities when the assays were conducted under standard optimal conditions. Measurement of *N*-acetylglutamate, however, the absolutely required allosteric activator of carbamoyl phosphate synthetase, revealed the effect of L-hydrazinosuccinate treatment. As shown in Table 2, the treatment induced a remarkable increase of the activator. The extent of the increase appears to correlate well with that of citrulline formation (Table 1), consistent with the regulatory function of carbamoyl phosphate synthetase [6].

Table 2. Effect of L-hydrazinosuccinate administration on *N*-acetylglutamate content and tryptophan dioxygenase activity of mouse liver*

Treatment of animals	*N*-Acetylglutamate [nmol·(g liver)$^{-1}$]	Tryptophan dioxygenase [nmol·min^{-1}·(g liver)$^{-1}$]
Control	25 ± 9	7.1 ± 1.5
L-Hydrazinosuccinate	62 ± 12	16.4 ± 2.1

* Liver samples were obtained from mice treated as described in Table 1 and processed for analysis of *N*-acetylglutamate content and assay of tryptophan dioxygenase as described in Materials and Methods. Values are means ± SD for three determinations, one from each animal.

Enhanced action of glucocorticoids induced by L-hydrazinosuccinate treatment. We previously observed a remarkable increase in the activity of mouse liver tyrosine aminotransferase several hours after administration of L-hydrazinosuccinate, and postulated that the increase is due to hormonal induction, probably involving glucocorticoids [2]. If the postulate is correct, other effects on amino acid metabolism including inhibition of protein synthesis in peripheral tissues and enhanced degradation of amino acids in liver may also occur. Thus, the elevated levels of glutamate and ammonia in liver as described above may be explained as results of glucocorticoid action.

To test the hypothesis, the effect of L-hydrazinosuccinate administration on tryptophan dioxygenase, one of the enzymes known to be rapidly induced by glucocorticoids was examined. Table 2 shows a marked increase in enzyme activity and supports the postulated action of glucocorticoids. Another attempt made to confirm hormone action was to determine the serum level of corticosterone, the major glucocorticoid in rodents. The results given in Table 3 indicate that the administration of the inhibitor in fact produced an elevated level of the hormone.

DISCUSSION

The present study indicates that the concentrations of citrulline in the liver and plasma rose enormously following administration of L-hydrazinosuccinate, which is reported to be a potent inhibitor of aspartate aminotransferase [1]. The elevated level of the amino acid did not last very long, being nearly at the control level in 24 hr.

The observation that the plasma urea level was not enhanced despite the presence of large amounts of citrulline suggested that the accumulation of the amino acid was produced by a blockade at some step of citrulline utilization in urea synthesis. One likely candidate for such a reaction seemed to be the formation of argininosuccinate, because this step is known to be the rate-limiting step of the urea cycle. Besides, inhibition by L-hydrazinosuccinate of argininosuccinate synthetase, which catalyzes the formation, seemed possible since the inhibitor is the *N*-amino derivative of aspartate which is required as a substrate of the enzyme. However, experimental results indicated that the enzyme was not affected by the inhibitor. Rather, the enzyme activity was increased 2-fold in 9 hr after L-hydrazinosuccinate injection. In addition, argininosuccinate lyase, catalyzing the next step of the urea cycle, appeared unaffected by the inhibitor. No good explanation was thus obtained by examination of enzyme activities. On the other hand, the observation that aspartate was decreased significantly in the liver suggests that there was not enough aspartate to support the conversion of citrulline to argininosuccinate. Furthermore, it is possible that the aspartate detected was mainly in the mitochondria and not in the cytosol where aspartate reacts with citrulline, although the present work provides no evidence for this possibility. The observed decrease in the aspartate level and increase of glutamate are at least partially explained as a result of inhibition of cytosolic aspartate aminotransferase by L-hydrazinosuccinate, for the cytosolic aspartate used for argininosuccinate formation is usually produced by the transamination between oxaloacetate and glutamate in the cytosol.

Table 3. Effect of L-hydrazinosuccinate administration on corticosterone content of mouse plasma*

Treatment of animals	Corticosterone (ng/ml)
Control	128 ± 25
L-Hydrazinosuccinate	270 ± 32

* Plasma samples were obtained from mice injected with L-hydrazinosuccinate (0.6 mmol/kg) 3 hr before and from control mice injected with saline immediatedly before and were analyzed as described in Materials and Methods. Values are means ± SD for three determinations, one from each animal.

Because the accumulation of citrulline appeared too prominent to be explained completely by the foregoing discussion based on the insufficiency of aspartate, we also examined the possibility of stimulated formation of citrulline. Experiments in fact indicated that the mitochondria of L-hydrazinosuccinate-treated mice had markedly enhanced activity of citrulline formation. Further experiments revealed that treatment with the inhibitor increased the amount of *N*-acetylglutamate, which is an absolutely required allosteric activator of the regulatory enzyme, carbamoyl phosphate synthetase. The elevated concentration of *N*-acetylglutamate was probably due to the presence of the enhanced concentrations of glutamate and ammonia as suggested by a previous report demonstrating the stimulation of *N*-acetylglutamate synthesis in isolated hepatocytes by glutamate and ammonia [15].

The finding of elevated concentrations of corticosterone in the serums of animals treated with the inhibitor provides an explanation for the increased levels of glutamate and ammonia. Probably, by the effect of the hormone in depressing protein synthesis in nonhepatic tissues, amino acids were metabolized in the liver, and transamination between the amino acids and 2-oxoglutarate produced glutamate, which in turn yielded ammonia on dehydrogenation.

In light of the potent inhibition of urea synthesis by aminotransferase inhibitors such as aminooxyacetate [13], the present observation that hydrazinosuccinate administration did not decrease significantly the plasma urea level appears to be an unexpected feature. Conceivably, urea synthesis was not inhibited markedly in the present experimental system *in vivo* because the inhibitory effect of the drug through its action on aspartate aminotransferase was mostly balanced by stimulatory effects of accumulated citrulline, glutamate and ammonia produced by increased degradation of amino acids.

A question remains as to how the glutococorticoid level was enhanced. For the present it may be assumed that the administration of the inhibitor induced the release of hormone because it imposed a kind of stress on the animals. The animals receiving

the drug appeared to be feeling some discomfort, being motionless compared with the control animals.

There is also a question of how the elevated citrulline level mostly returned to the control level in 24 hr while cytosolic aspartate aminotransferase continued to be severely inhibited [2]. One explanation is provided by the nearly 2-fold increase in the activity of argininosuccinate synthetase shown in Fig. 3. The enzyme is known to be induced by glucagon, whose secretion is stimulated by amino acids. Perhaps the increase of amino acids caused by the glucocorticoid indirectly induced the enzyme activity. Another explanation is that the elevated level of the glucocorticoid usually does not last long, although this was not determined in the present work. In agreement with the explanation, not only citrulline but most amino acids examined were found to be at nearly normal concentrations in liver 24 hr after the injection of the inhibitor (data not shown).

In conclusion, L-hydrazinosuccinate induced accumulation of citrulline through its specific effect of inhibiting aspartate aminotransferase and a rather nonspecific effect of stimulating the release of glucocorticoids. The accumulation of citrulline shown in this study may provide a useful model for citrullinemia.

The observed elevation of plasma tyrosine level (Fig. 2) in the hydrazinosuccinate-treated mice is difficult to explain. This seems to conflict with the prominent enhancement of tyrosine aminotransferase activity in the liver as previously reported [2]. Further studies are required to clarify this problem.

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