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Regulation of Orotic Acid Biosynthesis and Excretion Induced by Oral Glutamine Administration in Mice[1]

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Glutamine, the most abundant amino acid in blood and tissues, is degraded by the renal and splanchnic tissues, especially the small intestinal mucosa. Due to the activity of glutaminase, it may be broken down in these tissues and contribute to ammoniagenicity. Glutamine, either directly or through ammonia production, may act as a nitrogenous source for pyrimidine biosynthesis. We have evaluated the effect of glutamine on orotate metabolism in mice, by gavaging (ig) L-glutamine, 1.0 to 4.0 mmol/100 g of body wt/day, during 6 weeks of experimentation. Glutamine at doses of 2.5 to 4.0 mmol/100 g of body wt caused a significant increase in plasma ammonia and urinary orotate. The regulation of the orotic acid biosynthesis and excretion was studied by testing the effects of various inhibitors in mice force-fed with glutamine (4 mmol/100 g of body wt, ig). The orotic aciduria was insensitive to acivicin (1 and 5 mg/100 g of body wt, ip), a specific inhibitor of the cytoplasmic carbamyl phosphate synthetase-II, thus pointing toward the mitochondrion as the principal source of carbamyl phosphate. Cycloheximide (15 and 100 mg/kg of body wt, ip) caused a significant decrease in urinary orotate indicating that the induction of orotate synthesis by glutamine may be associated with the translation of a specific protein. However, orotate excretion was significantly decreased by *N*-(phosphonoacetyl)-L-aspartate (PALA) (5 mg/100 g of body wt, ip) due to its inhibitory effect on the aspartate transcarbamylase activity. There was a significant increase of urinary orotate following ingestion of adenine supplemented diets (0.1% and 0.2%), suggesting the blockage of the utilization of orotate for nucleotide biosynthesis by glutamine. Since orotate synthesis may also be influenced by ornithine metabolism, we evaluated the effect of glutamine administration on various ornithine-metabolizing enzymes. There was a decrease in hepatic ornithine decarboxylase activity with no change in hepatic ornithine aminotransferase activity following the administration of glutamine. This observation indicates that an increased metabolic utilization of ornithine is not responsible for the increase in orotate excretion, which may be caused principally through an effect of glutamine on mitochondrial carbamyl phosphate synthesis.

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Glutamine is the most abundant amino acid in the blood and tissues and accounts for nearly two-thirds of the intracellular amino acid pool in the skeletal muscle (1,2). It is an important vehicle for transport of nitrogen from muscle to renal and splanchnic tissues, and a major respiratory fuel for various tissues, especially the intestinal cells (3), the activated lymphocytes (4), and the kidneys (5). Muscle glutamine may be considered as a labile nitrogen pool that could participate in acute changes in the whole body nitrogen balance. However, the major site of utilization of glutamine in the nonhepatic splanchnic bed is the mucosa of the small intestine (3). Due to the activity of glutaminase, the endogenous and exogenous glutamine may be broken down in the intestine and the liver and contribute to ammoniagenicity. Several factors such as hormonal modifications (glucagon, glucocorticoids) (6), ammonia concentration (7,8), high protein diets (8,9), chronic metabolic acidosis (7,10,11), and bicarbonate administration (12) can influence glutamine and ammonia metabolism in the hepatic periportal area.

Rudman *et al.* (13) have presented evidence that only a few amino acids, including glutamine, have ammoniagenic properties when administered in the diet. In fact, glutamine was considered to be fourth in importance among amino acids after glycine, serine, and threonine, from the point of view of the oral ammoniagenic potency. As shown in *in vitro* studies, glutamine, either directly or through ammonia production, may act as a nitrogenous source for pyrimidine biosynthesis (14,15). However, there are no reports in the literature concerning the effect of oral administration of glutamine on pyrimidine synthesis. In the work of Hatchwell and Milner (16) on the effects of various amino acids on orotic aciduria, glutamine was injected. We planned our experimental studies based on an oral administration of glutamine because (a) glutamine is one of the principal components of dietary amino acids; (b) it may be utilized in the intestinal mucosa; and (c) a minimal quantity of this amino acid appears in the portal blood during the digestion (17), which could be taken up by the hepatocytes through a system of N transport (18).

Our first objective was to investigate the effects of chronic oral administration of various doses of glutamine on orotate (OA) metabolism, which may have an interrelationship with the ammoniagenic properties of glutamine in mice. The second objective was to identify the source of carbamyl phosphate (CP) used for OA synthesis, by studying the effect of acivicin, a specific inhibitor of the cytosolic CPS-II. This enzyme is directly dependent on glutamine as the source of ammonia for carbamyl phosphate synthesis, whereas the mitochondrial CPS-I can use free ammonia arising from deamination of various amino acids. The third objective was to study the mechanism by which oral glutamine enhances OA biosynthesis and its regulation in mice through the effects of various general and specific inhibitors. Since orotate synthesis may also be influenced by ornithine metabolism through ornithine-utilizing enzymes, we evaluated the effects of oral glutamine administration on hepatic ornithine decarboxylase (ODC) (EC 4.1.1.17) and ornithine aminotransferase (OAT) (EC 2.6.1.13) activities. This study was considered important in view of the recent observation that high levels of OA caused nucleotide pool imbalance (19,20), inhibited hepatocyte cell proliferation *in vitro* and *in vivo* (21–23) and promoted carcinogenesis in many organs including liver,

duodenum, mammary glands, and possibly in pancreas, kidneys, and lungs (24–26).

Our results indicate that an oral load of glutamine contributes to increased blood ammonia and urinary orotate, that the source of CP for orotate synthesis seems to be principally mitochondrion, and that the activities of ornithine-metabolizing enzymes were not increased following oral glutamine administration.

MATERIALS AND METHODS

The Effects of Oral Glutamine on Blood Ammonia and Urinary Orotate

After adaptation to a semi-synthetic basal diet (Purina Chow, Richmond, IN), containing at least 22% protein, and to a daily cycle of 12 h light and darkness, male Swiss–ICR mice, 10 weeks of age, weighing on average 36 g, were selected. Animals (n = 5 to 9 per group) were fed *ad libitum* with the same basal diet. The quantity which remained after consumption was weighed. Control groups were gavaged (ig), between 10:00 am and 12:00 noon, with 1.0 ml of 0.9% NaCl, while experimental groups were force-fed (for 6 weeks) with glutamine (ICN Biochemicals, Cleveland, OH) from 1.0 to 4.0 mmol/100 g of body wt dissolved in 0.9% NaCl. At the end of 6 weeks, mice were sacrificed by removing blood through cardiac puncture. Blood samples were thereafter used for plasma ammonia estimation (27). Mice were weighed, from Days 0 to 42, every week. Urine collections were done for periods of 24 h, while mice were individually placed in metabolic cages (Maryland Plastics Inc., Federalsburg, MD) every 7 days, for urinary OA estimation (28,29), expressed on a per milligram creatinine basis in order to correct for variations due to body size. This method uses a nonbrominated blank with each sample tube to correct for interfering substances. Creatinine was determined by ultramicroadaptation of the method of Folin and Wu (30).

The Effect of Acivicin on Orotate Synthesis

In order to identify the source of CP for OA synthesis, we evaluated the specific effect of acivicin on the cytosolic pyrimidine pathway. Thirty minutes prior to the administration of glutamine, mice were pretreated with acivicin (1 and 5 mg/100 g of body wt, ip) (Sigma Chemical Co., St. Louis, MO).

The Effect of General and Specific Inhibitors on Orotate Synthesis

In order to determine the possible mechanisms for the effect of oral glutamine on OA biosynthesis and its regulation, we evaluated the general and specific effects of various inhibitors. Thirty minutes prior to the administration of glutamine, mice were pretreated with cycloheximide (15 and 100 mg/kg of body wt, ip) (Sigma Chemical Co.) or *N*-(phosphonacetyl)-L-aspartate (PALA, 5 mg/100 g of body wt, ip) (gift from Dr. D. S. R. Sarma). Mice were then gavaged with 0.9% NaCl or 0.9% NaCl containing glutamine (4.0 mmol/100 g of body wt, ig) during 48 h. In another set of experiments, adenine (0.1 and 0.2%) (Dyets Inc., Bethlehem, PA) was given in the diet for 7 days.

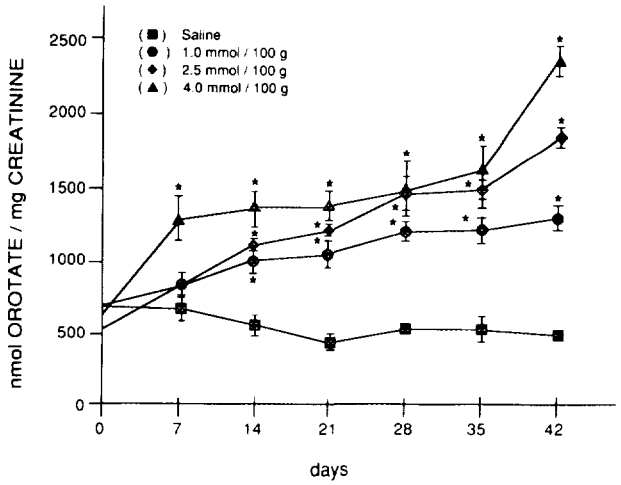

FIG. 1. The progressive effects of different doses of glutamine on urinary orotate levels in mice as seen in 24-h samples collected every week for 6 weeks. Mean values ± SEM ($n = 7$). * $P < 0.01$; nonparametric test of Mann–Whitney; significantly different from the saline group.

Determination of the Hepatic ODC and OAT Activities and Measurement of Total Protein

Liver samples were excised from mice treated with various doses of glutamine (1–4 mmol/100 g of body wt, ig) for 6 weeks. In order to assay the ODC activity, we used a micro method previously described by Beaven *et al.* (31). Liver samples were individually placed in Hepes buffer (50 mM, pH 7.4) (Sigma Chemical Co.). The samples were homogenized and centrifuged in a refrigerated centrifuge at 10,000*g* for 15 min. Ornithine decarboxylase activity in supernatants derived from 10% liver homogenates was determined by the amount of $^{14}CO_2$ released from L-[1-^{14}C]ornithine (sp. act: 54 mCi/mmol) (ICN Radiochemicals Inc., Irvine, CA). Total protein content was determined by the method of Bradford (32).

Ornithine aminotransferase (OAT) activity was measured from the same liver samples, according to the method described by Peraino and Pitot (33).

Statistical Analyses

Data from all the experiments are expressed as means ± SEM (standard error of the mean). The Mann–Whitney rank sum test, unpaired Student *t* test, and Pearson correlation coefficient were used when appropriate. *P* values of ≤ 0.05 were considered significant.

RESULTS

Progressive and Cumulative Effects of Glutamine on Orotate Synthesis

Figure 1 represents the progressive effect of different doses of glutamine in respect of time of experimentation, based on 24-h samples collected every week for 6 weeks, while Table 1 indicates the cumulative effect of glutamine on orotate

TABLE 1
Plasma Ammonia and Urinary Orotate in Mice after 6 Weeks of Treatment with Glutamine[a]

Glutamine (mmol/100 g)	Final weight (g)	Plasma ammonia (μmol N/liter)	Urinary orotate[b] (nmol/mg creatinine)
0	35 ± 0.8	131 ± 9	495 ± 28
1.0	36 ± 0.6	159 ± 18	1321 ± 88**
2.5	36 ± 1	258 ± 13**	1843 ± 101**
4.0	35 ± 1	280 ± 12**	2387 ± 100**

[a] Data are means ± SEM of seven samples. Mice were gavaged with 0.9% NaCl or glutamine (1.0 to 4.0 mmol/100 g of body wt, ig) suspended in NaCl (0.9%).
[b] Urine collections and OA estimation were done for periods of 24 h at the end of 6 weeks.
** $P < 0.01$; significantly different from 0.9% NaCl group (Mann–Whitney rank sum test).

metabolism, at the end of 6 weeks of continuous administration. The urinary excretion of OA was significantly increased at all glutamine concentrations as compared to the control groups (Fig. 1). The increase in plasma ammonia and urinary orotate was proportional to the dose of glutamine administered (Table 1).

Effect of Acivicin on Orotic Aciduria

Figure 2 shows the effects of different (ip) doses of acivicin on the synthesis and excretion of OA caused by glutamine. Acivicin treatment did not significantly alter the OA excretion as compared to control group.

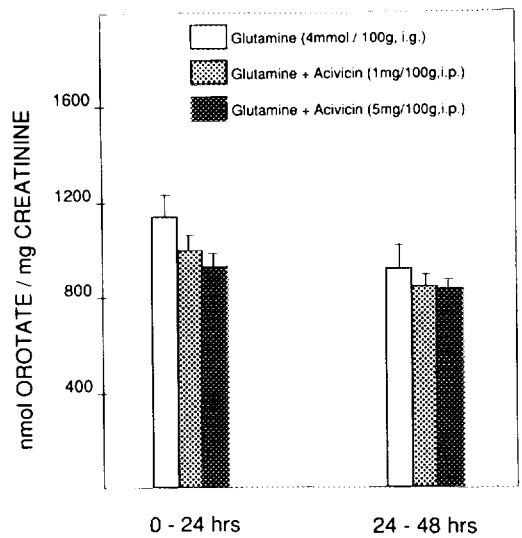

FIG. 2. Effects of different doses of acivicin on the urinary excretion of orotic acid induced by glutamine. Mean values ± SEM ($n = 7$), not significantly different from the glutamine group (non-parametric test of Mann–Whitney).

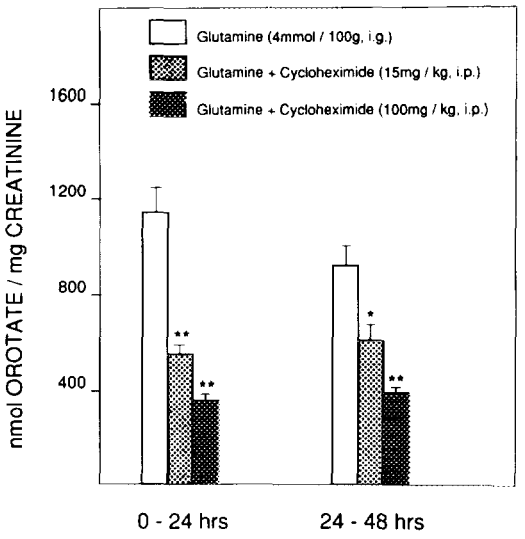

FIG. 3. Effects of different doses of cycloheximide on the urinary excretion of orotic acid induced by glutamine. Mean values ± SEM ($n = 7$). * $P < 0.05$, ** $P < 0.01$; nonparametric test of Mann–Whitney; significantly different from the glutamine group.

Effect of Cycloheximide on Orotic Aciduria

Figure 3 indicates the effects of different (ip) doses of cycloheximide on the synthesis and excretion of OA induced by glutamine. The administration of cycloheximide significantly decreased the urinary excretion of OA within 24 h. A similar effect continued for up to 48 h.

Effect of PALA on Orotate Excretion

A significant reduction of urinary orotate levels following PALA administration was observed. In Table 2, a comparison with various other inhibitors is also shown.

Effect of Adenine on Orotate Excretion (Table 3)

Mice treated with adenine (0.1 and 0.2%) exhibited a large increase of urinary orotate. A significant positive correlation ($r = 0.84$; $P < 0.01$) between urinary OA and adenine diets consumed was also observed.

ODC and OAT Activities in Liver

The values of hepatic ODC and OAT activities for different groups of experimental mice are given in Table 4. The hepatic ODC activity was lower, whereas the hepatic OAT activity was normal following the oral administration of glutamine.

DISCUSSION

In 1973, Rudman *et al.* (13) provided evidence that certain amino acids act as ammoniagenic substrates. This study showed that, in cirrhotic patients as compared

TABLE 2
Effects of Cycloheximide, Acivicin, and PALA[a] on Urinary Orotate Levels Following the Administration of Glutamine

Treatment[c]	Urinary orotate[b] 0–24 h	24–48 h
0.9% NaCl	378 ± 40	309 ± 31
Glutamine	1156 ± 102*	927 ± 58*
Glutamine + cycloheximide	359 ± 28**	383 ± 33**
Glutamine + acivicin	928 ± 79	846 ± 53
Glutamine + PALA	325 ± 37**	232 ± 9**

[a] PALA refers to *N*-(phosphonacetyl)-L-aspartic acid, an inhibitor of aspartate transcarbamylase.
[b] Expressed as nanomoles of excreted orotate per milligram of creatinine.
[c] Mice were gavaged with 0.9% NaCl or 0.9% NaCl containing glutamine (4.0 mmol/100 g of body wt, ig). In appropriate groups, mice were pretreated with cycloheximide (100 mg/kg of body wt, ip), acivicin (5 mg/100 g of body wt, ip), and PALA (5 mg/100 g of body wt, ip), 30 min prior to the administration of glutamine. Urine collections and OA estimation were done for periods of 24 and 48 h. Data are means ± SEM of 7 mice.
* $P < 0.01$ (Mann–Whitney rank sum test); significantly different from 0.9% NaCl group.
** $P < 0.01$ (Mann–Whitney rank sum test); significantly different from glutamine group.

to normal subjects, ammonia production in the body is more elevated for certain amino acids including glutamine. It was concluded that ammonia is produced directly within host tissues by deamination or deamidation and that this pathway is strongly dependent on the type of dietary amino acids. Although glutamine is a major constituent of endogenous and dietary proteins, a minimal quantity of this amino acid appears in the portal blood during the digestion (17). The metabolism of glutamine and its deamidation in the small intestine can release am-

TABLE 3
Effect of Adenine on Orotate Metabolism in Mice Force-Fed with Glutamine for 7 Days[a]

Adenine content (%)	Consumed diet[b] (g)	Urinary orotate[c] (nmol/mg creatinine)
0	25 ± 3	2129 ± 206
0.1	38 ± 4**	3333 ± 764*
0.2	33 ± 2**	4178 ± 1283**

[a] Data are means ± SEM of seven samples. Mice were gavaged with glutamine (4.0 mmol/100 g of body wt, ig) dissolved in NaCl (0.9%).
[b] Weight of the consumed diet containing 25% protein; Dyets Inc.
[c] Urine collections and OA estimation were done for periods of 24 h.
* $P < 0.05$, ** $P < 0.01$; significantly different from control group (Mann–Whitney rank sum test).

TABLE 4

Induction of Hepatic Ornithine Decarboxylase and Ornithine Aminotransferase Activities with Glutamine Force-Gavaged in M

Glutamine (mmol/100 g)	ODC activity[b] (pmol/h/g of tissue)	OAT activity[b] (nmol/min/g of tissue)	ODC sp ac (pmol/h/mg protein)	(nmo
0	989 ± 60	1267 ± 26	5.5 ± 0.3	
1.0	904 ± 51	1422 ± 79	5.0 ± 0.3	
2.5	769 ± 54	1323 ± 32	3.5 ± 0.3	
4.0	450 ± 43*	1159 ± 104	2.5 ± 0.2*	

[a] Data are means ± SEM of five to nine samples. Mice were gavaged with 0.9% NaCl or glutamine (1.0 to 4.0 mmol/100 dissolved in NaCl (0.9%).

[b] ODC and OAT activities refer to the activities of hepatic ornithine decarboxylase and ornithine aminotransferase after 0.9% N treatments.

* $P < 0.05$; significantly different from 0.9% NaCl group by using unpaired Student t test.

monia into the portal system (34). The ammonia may be increased after food ingestion (35), due to the metabolism of various amino acids present in the intestinal sink. Ammonia can also be produced partially from other amino acids in the basal diet either directly by their degradative pathway or indirectly by a transamination reaction with α-ketoglutarate to form glutamate which is further deaminated (13). The distribution of glutamine through organs following dietary feeding, particularly in the intestinal sink, allows for its utilization as a preferential source of energy, even in the presence of glucose (1). Excessive ammonia can be trapped by the liver, via the portal blood, and leads to an increased carbamyl phosphate (CP) level and excessive orotic aciduria (8,14,36). The quantity of formed glutamate could then be converted to aspartate and other derivatives by transamination reactions, including the formation of alanine, proline, ornithine, and citrulline (1,3). Fico *et al.* reported that there is a significant positive correlation between plasma ammonia concentrations and urinary OA levels (37). It has been suggested that the OA production mainly (over 80%) results from mitochondrial CP, which is shunted to the cytosolic pyrimidine pathway (14). In the present study, our results provide evidence of an interrelationship between ammonia and OA levels (Table 1). The association between the doses of glutamine administered and the increase of orotic aciduria is consistent with this interpretation. The main sources of plasma ammonia in the treated mice (Table 1) could be the amide groups of glutamine and the amino groups of glutamate and their derivatives. Data presented in Figure 1 reveal that long-term glutamine administration can affect OA levels in mice in a progressive manner.

The pretreatment of experimental animals with acivicin, a potent inhibitor of cytosolic CP synthetase (38), did not alter the OA excretion (Fig. 2). Our results suggest that the mitochondrial carbamyl phosphate synthetase (CPS-I) is involved and indicate mitochondria as the source of CP. In addition to the negative effect of acivicin on the synthesis of OA in mice treated orally with glutamine, our results indicate a relative inactivity *in vivo* of the cytosolic carbamyl phosphate synthetase (CPS-II). Glutamine is considered as the essential source of ammonia used by the CPS-II for the formation of CP. In our studies, the production of CP was not inhibited by acivicin and correspondingly was not influenced by the levels of glutamine consumed, due to the primary degradation of this amino acid in the small intestine. Finally, the channeling of mitochondrial CP toward the cytosolic pyrimidine pathway is an indication of the site from which CP originates and where it is used for orotate synthesis in mice. The data also support the other reports in the literature (16,39–41) that the induction of orotate synthesis is not unique to one amino acid, but may be a property of all ammoniagenic amino acids, including glutamine, acting on the mitochondrial CP synthesis.

The pretreatment with cycloheximide (39), before oral glutamine administration, decreased the urinary excretion of OA to one-third within 24 h (Fig. 3). These data (see Table 2 also) suggest that the synthesis of OA induced by glutamine is controlled by the synthesis of a protein; this effect revealed a translational dependent event. A reduction of urinary orotate was seen (Table 2), following PALA administration, an inhibitor of aspartate transcarbamylase (38,42). These data suggest that the mitochondrial CP is converted in the cytosol by the cytosolic

aspartate transcarbamylase to form carbamyl aspartate (CA) and revealed the blockage of the orotate formation. The increased synthesis and excretion of OA, following adenine-supplemented diets (0.1 and 0.2%) (Table 3), suggest, furthermore, an inhibition of the utilization of OA (43) in the formation of uridine monophosphate (UMP). The results (Table 3) indicate that the adenine molecule, an agent which competes with orotic acid for 5-phosphoribosyl-1-pyrophosphate, and inhibits its metabolic conversion to orotidylic acid and uridilic acid, increased the excretion of urinary orotic acid. These results suggest that a decreased metabolic conversion of orotic acid to orotidylic acid may be a significant contributing factor for the increased excretion of urinary orotic acid.

The hepatic ODC activity decreased following the administration of glutamine (Table 4). This observation indicates that the regulatory step in the synthesis of OA from glutamine does not involve the lack of ornithine which would normally regulate the chaneling of CP toward the cytosolic pyrimidine pathway. An induction of ODC activity could decrease the levels of cytoplasmic ornithine such that CP is then available for OA synthesis. Our results discount the possibility that the induction of ODC activity and OA synthesis by certain amino acids are mediated through a common mechanism (44). However, it has been demonstrated that glycine can induce the expression of certain cell cycle dependent genes, including that of ODC. Consequently the orotic aciduria seen in glycine-supplemented animal models could be partly caused by the induction of ODC and an increased utilization of ornithine through this pathway. It is also known that ODC activity is primarily induced by those neutral amino acids (glycine, asparagine, glutamine, α-aminoisobutyric acid (AIB), *N*-methyl-AIB, proline, and serine) which utilize the Na^+-dependent A and N systems. ODC activity is not induced by other neutral amino acids (phenylalanine, leucine, isoleucine, and valine) which are transported primarily by the Na^+-independent L system (45). The acidic amino acids (X-system), including the L-aspartic and L-glutamic acids, do not induce ODC activity (45). The degradation of orally administered glutamine to form glutamate, aspartate, and other nitrogenous derivatives in the intestinal tract could explain the tendency for a fall in hepatic ODC activity seen in the present study (Table 4).

OAT is a mitochondrial enzyme that catalyzes the reaction whereby the amino group of ornithine is transferred to α-ketoglutarate to yield glutamate and glutamate-γ-semialdehyde (33). In the present study (Table 4), the maintenance of hepatic OAT activity following the administration of glutamine suggests no changes in the levels of ornithine and reveals that its increased utilization is not responsible for the increase in OA excretion.

The present report notes that glutamine can cause a significant increase of orotate, which has been reported to promote carcinogenesis in the liver, as well as in several other organs (24–26). The significance of our study is that it raises several important questions to be addressed in the future. These are the following: (a) Are very high concentrations of glutamine in amino acid solutions indicated for parenterally fed patients? (46,47) (b) Can glutamine singly or in concert with other amino acids induce a significant increase of orotate synthesis at the clinical level? (c) If so, is there a possibility of a complete mixture of amino acids used

in the clinical parenteral nutrition causing metabolic perturbation related to increased orotate, and eventually to promote tumors in newborn infants fed through intravenous regimens over extended periods? (d) These results also raise the question whether high glutamine diets may favor the growth of tumor cells by simply providing the nutrients or also by inducing a promotional mechanism associated with progression through cell proliferation (48,49).

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