Evaluation of L-Asparagine Metabolism in Animals and Man

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SUMMARY

A rapid, sensitive, and specific method for the determination of L-asparagine and L-aspartic acid has been developed based on an enzymatically coupled oxidation of reduced pyridine nucleotide. With this spectrophotometric or fluorimetric method, the concentration of these amino acids has been determined in normal plasma, erythrocytes, tumors, urine, and cerebrospinal fluid. A modification of this method permitted measurement of 0.001 i.u. L-asparaginase. A variety of disease states in humans had relatively little effect on the levels of L-asparagine in plasma. It was shown that hepatectomy caused a two-fold elevation of plasma levels of L-asparagine in rats, but that hypophysectomy and dietary deprivation did not produce significant changes in rodents. In human subjects, however, a large oral dose of L-asparagine created a sustained elevation of the level of L-asparagine in the plasma. In an attempt to reduce plasma concentrations of L-asparagine in humans by hemodialysis, it was found that, despite extensive loss in the dialysate over a period of 6 hr, the plasma concentration was essentially unchanged. L-asparaginase treatment of a dog with lymphosarcoma caused marked objective improvement and the expected elimination of plasma L-asparagine. The levels of this amino acid in erythrocytes, urine, and tumor tissue, however, were slow to follow the changes in plasma concentration and returned to normal values much more rapidly.

INTRODUCTION

It has been shown that certain lines of malignant cells in culture require L-asparagine for growth (8, 15) and that the enzyme L-asparaginase from various sources causes inhibition of the growth of selected tumors (3, 10, 12, 19). Further investigation of the metabolism of L-asparagine and of the effects of L-asparaginase on the rate of tumor growth required a rapid, sensitive, and specific assay for L-asparagine. The several methods currently used either require large amounts of sample (4, 20, 22) or are time consuming and do not lend themselves to multiple assays (21). In the present report, a spectrophotometric assay for N-acetyl-L-aspartic acid (13) has been modified to permit its application to the analysis of L-asparagine and L-aspartic acid in tissues and body fluids. The method involves a sequence of enzymic reactions which result ultimately in the oxidation of NADH. With this method it has been possible to determine the concentration of L-asparagine in various tissues, to follow the disposition of L-asparagine in animals and human subjects under various conditions, and to assess the deficiency of L-asparagine in plasma and tissues after the administration of L-asparaginase from Escherichia coli.

MATERIALS AND METHODS

Materials. L-Asparagine monohydrate, L-aspartic acid, α-ketoglutaric acid, and Tris were obtained from Sigma Chemical Co., St. Louis, Mo. NADH was obtained from Calbiochem, Los Angeles, Calif. The enzyme glutamic-oxaloacetate transaminase (180 i.u./mg) and malic dehydrogenase (720 i.u./mg), isolated from pig heart, were obtained from Boehringer und Soehne GmbH, Mannheim, Germany. L-Asparagine was freed of the L-aspartic acid contaminating most commercial preparations by chromatography through a column (2 x 10 cm) of Dowex 1-X4 (formate) (200 to 400 mesh). Even at 4°C such purified solutions of L-asparagine were slowly hydrolyzed to L-aspartic acid and ammonia and were freshly prepared. The L-asparaginase (EC 3.5.1.1) E.C.-2 used in these studies had a specific activity of approximately 150 i.u./mg protein and was designated TAD6A by the manufacturer, E. R. Squibb and Sons, New Brunswick, N. J.

The 6C3HED lymphoma was maintained by subcutaneous transfer in C3H mice. The L5178Y and P815Y leukemias were propagated in the ascitic form in AKD2F1 mice. Unless otherwise indicated, all animals were maintained on a diet of Purina laboratory chow and tap water ad libitum. Hypophysectomized rats were purchased from Charles River Breeding Laboratories, Inc., Boston, Mass.; albino rats were partially hepatectomized under ether anesthesia and from rats by aspiration after Nembutal anesthesia, and from rats by decapitation. Tubes containing blood were iced and the plasma was separated at 4°C in an International clinical centrifuge at 2500 rpm for 15 min. Erythrocytes were either processed directly or washed with 0.9% NaCl solution at 4°C prior to being processed. The separated plasma was pipetted into 10-ml polycarbonate centrifuge tubes and heated in a boiling water bath for 20 min. Apart from an initial 1:1

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dilution with distilled water, erythrocytes were deproteinized in a manner identical to that used for plasma.

Other tissues were excised, weighed, and homogenized with a Teflon pestle in a Potter-Elvehjem apparatus at 0° in 2 to 7 volumes distilled water. The homogenates were processed in polycarbonate tubes as described for plasma. After heating, the congealed samples of plasma erythrocytes or tissues were cooled and centrifuged at 105,000 X g at 4° for 1 hr. Clear supernatant fluid was withdrawn and either stored at −20° or assayed immediately.

For the determination of L-aspartic acid and L-asparagine in urine, aliquots that had been kept at 0° were processed by boiling to destroy any urinary enzymes and centrifuged at 0 to 4° to remove any sediment. Because of the high intrinsic absorbance of such samples at 340 nm, only small aliquots (0.1 to 0.2 ml) could be analyzed directly. A modification of this method, applicable only to the assay of L-asparagine, was used with highly pigmented urines or when the concentration of L-asparagine was low. Pasteur pipets were plugged with glass wool and half filled with Dowex 1-X4 (formate), 200 to 400 mesh, column (4 x 80 mm). Three-column volumes of urine were passed through the resin-filled pipets and the effluents were discarded. The 4th effluent from this column, a clear and colorless solution, was used for the assay of L-asparagine.

RESULTS

Evaluation of Assay Method. The spectrophotometric assay for L-asparagine and L-aspartic acid was done as presented in Table 1, and followed the reaction sequence outlined in Chart 1. Absorbance at 340 nm was measured at 3 stages and the concentration of L-aspartic acid and L-asparagine calculated according to the formula:

\[
\text{L-Aspartic acid} \text{ nmoles/ml} = \text{dilution factor} \times \frac{20}{0.115} \times \frac{A_1 - A_2}{\text{volume of sample (ml)}}.
\]

For L-asparagine concentration, the same formula is used, except that \(A_2\) replaces \(A_1\), \(A_3\) replaces \(A_2\), and an appropriate dilutional correction is used.

Dilutional factor refers to the dilution of the sample with water in processing; dilutional correction refers to the volumetric dilution of the reaction mixture when enzyme was added; and the term \(\frac{20}{0.115}\) represents 20 nmoles L-aspartic acid or L-asparagine, which, in the 1.1-ml reaction volume, cause a \(\Delta A\) at 340 nm of 0.115. Standard curves for L-asparagine and L-aspartic acid are presented in Chart 2 A.

The purified preparations of E. coli L-asparaginase (E.C.-2) used in these studies were found to be devoid of NADH oxidase. Although other substrates for L-asparaginases are known, notably D-asparagine in the case of the guinea pig enzyme (22), their enzymatic hydrolysis would not form L-aspartic acid, for which the pig heart transaminase preparation has a highly specific requirement. The transaminase will, however, accept cysteinesulfinic acid instead of L-aspartic acid (11), but the product of such a transamination, sulfinylpyruvic acid, cannot be reduced by malic dehydrogenase.

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<th>Reagent</th>
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<tr>
<td>Sample or standard containing L-aspartic acid or L-asparagine</td>
<td>0.005-0.14 μmole</td>
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<tr>
<td>α-Ketoglutaric acid</td>
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<tr>
<td>NADH</td>
<td>0.2 μmole</td>
<td>0.1</td>
</tr>
<tr>
<td>Tris buffer, pH 8.0</td>
<td>50.0 μmole</td>
<td></td>
</tr>
<tr>
<td>GOT</td>
<td>0.8 i.u.</td>
<td></td>
</tr>
</tbody>
</table>

Record absorbance at 340 nm (Absorbance 1) after 30 min at room temperature, record absorbance at 340 nm (Absorbance 2) after 30 min at room temperature, record absorbance at 340 nm (Absorbance 3)

\(\Delta A\) represents 20 nmoles L-aspartic acid or L-asparagine, which, in the 1.1-ml reaction volume, cause a \(\Delta A\) at 340 nm of 0.115. Standard curves for L-asparagine and L-aspartic acid are presented in Chart 2 A.
Glutamic acid in 10-fold excess did not interfere with the enzymic assay.

The specificity of the reaction is lost in the presence of extracts that consume NADH, such as pyridine nucleotide-linked dehydrogenases, or systems that will generate oxalacetic acid. Thus it is necessary to remove these interfering enzymic activities by denaturation of enzymes in the sample to be assayed. In this respect, appreciable malic dehydrogenase activity has been found in neutralized 5% perchloric acid extracts of certain tissues, notably 6C3HED tumors. The presence of this enzyme will invalidate the L-aspartic acid assay, but not the assay for L-asparagine.

In an experiment to test the reproducibility of the method, a commercial sample of horse serum was assayed repeatedly. In triplicate determinations, the values for L-aspartic acid were 43, 43, and 44 nmoles/ml, and for L-asparagine the values were 4.0, 3.7, and 4.0 nmoles/ml. When 52 nmoles L-aspartic acid and 45 nmoles L-asparagine/ml were added prior to processing the samples, the recovery in 8 determinations ranged from 93 to 100% for L-aspartic acid and from 97 to 105% for L-asparagine.

To evaluate the extraction of asparagine from plasma and tissues, alternative techniques of protein precipitation were used with 3 independent determinations in each method. The L-asparagine concentration in human plasma prepared by the boiling method was 34 nmoles/ml plasma. Protein removal by 5% trichloroacetic acid and subsequent extraction of the acid with ether gave 35 nmoles/ml plasma; precipitation with 0.5 M perchloric acid and subsequent neutralization with KOH gave 33 nmoles/ml plasma. Similarly, analyses of the L-asparagine content of erythrocytes indicated 91 nmoles/ml by the boiling method, 88 nmoles/ml by trichloroacetic acid precipitation, and 83 nmoles/ml by perchloric acid precipitation.

To increase the sensitivity of the assay to accept 0.02 ml normal plasma, the oxidation of NADH can be followed fluorometrically as described for other pyridine nucleotide reactions (7) (Chart 2F). There is an acceptable correlation between the spectrophotometric and fluorometric assays (Chart 2C). The decrease in fluorescent emission, in arbitrary units, was found to be within ±2.5% on successive days for a given amount of L-asparagine.

It was also possible to adapt the sequential spectrophotometric assay for L-asparagine and L-aspartic acid to the measurement of low levels of L-asparaginase. In a volume of 2.2 ml were mixed 0.36 μmole L-asparagine, free of L-aspartic acid; 7.2 i.u. malic dehydrogenase; 36 i.u. GOT; 200 μmoles Tris, pH 8; and 2.0 μmoles α-ketoglutarate. At 0 time, 20 μl sample containing approximately 0.020 i.u. enzyme was added to a cuvet that had been warmed to 37°. The reaction rate at 37° was then measured in a recording spectrophotometer with a thermostatic control. From the slope of this reaction the unitage of L-asparaginase can be calculated. With this method the sensitivity of this assay is approximately 0.005 i.u. (ΔA340 nm of 0.003/min). In a similar assay, L-asparagine hydrolysis was allowed to proceed at 37° for 60 min and the reaction was terminated with perchloric acid. L-Aspartic acid could then be measured spectrophotometrically in the neutralized reaction mixture. As little as 0.00006 i.u. L-asparaginase was measurable in this manner (ΔA340 nm 0.025/60 min of preincubation).

**Determination of L-Asparagine Levels in Diverse Conditions.** These analytical methods have permitted studies of the effects of various physiological factors on the normal levels of L-asparagine in plasma and tissues. The concentration of L-asparagine in plasma from normal mice, rats, dogs, and man are presented in Table 2. The concentration of L-asparagine in normal human cerebrospinal fluid (3.7 to 8.3 nmoles/ml) was consistently 0.2 to 0.1 that found in the plasma. The concentration in isolated leukocytes from 6 patients with myeloproliferative disorders and high peripheral leukocyte counts ranged from 82 to 300 nmoles/g cells. Analysis of 6C3HED tumors in C3H mice indicated a concentration of 91 to 366 nmoles/g, a range similar to the value reported by Broome (125 nmoles/g) (5).

**The Effect of a Variety of Human Diseases on the Concentration of L-Asparagine in Plasma.** Samples were taken between 6 and 9 a.m. from fasting patients. In this group of 65 patients, including those with hepatic failure, chronic lung disease, renal failure, collagen diseases, malnutrition, diabetes mellitus, and arteriosclerotic cardiovascular disease, as well as a wide variety of human neoplastic disease, the plasma levels of L-asparagine did not differ by more than 10 nmoles L-asparagine/ml from the range of values obtained in normal subjects. In 2 individuals, however, plasma concentrations of L-asparagine significantly outside the normal range.
were observed. One patient with acute histiocytic leukemia had 24 nmoles/ml plasma and 1 of a group of 5 women with metastatic carcinoma of the breast had an elevated level of this amino acid (100 to 135 nmoles/ml) in 5 separate determinations. Members of this patient’s family had concentrations of L-asparagine in the plasma consistently above the normal range. More detailed studies of this family are in progress. It would appear then, that homeostatic control of the concentration of L-asparagine in plasma is achieved to a remarkable degree in most human subjects with highly diverse disease states.

A standard synthetic diet (9) prepared with acid-hydrolyzed casein (free of L-asparagine by assay) was fed to adult male C3H mice for 21 days. The L-asparagine concentration in the plasma of 8 mice on the experimental diet (22 nmoles/ml plasma) was not significantly reduced from that of their counterparts (25 nmoles/ml) maintained on laboratory chow. Endogenous synthesis, therefore, was able to compensate adequately for this dietary deprivation.

In an extension of these dietary studies to humans, the partition of L-asparagine p.o. in extracellular body fluids of hydrated but fasted human subjects, as well as its passage into erythrocytes, was determined (Chart 3). The plasma clearance curve indicated a biphasic clearance of this unphysiological load. Erythrocytes did not readily equilibrate with the marked elevation of plasma L-asparagine; approximately 100 min were required for the concentration in erythrocytes to reach double its original level. In 1 of the 2 subjects studied, a very slight elevation of plasma L-aspartic acid accompanied the peak in L-asparagine; in both, approximately 0.0001% of the dose was recovered in the urine as L-aspartic acid. Ingestion of 1 mmole D-asparagine/kg body weight failed to increase significantly plasma levels of L-asparagine in 1 subject. The concentration of the essential and unrelated amino acid, L-tryptophan, in the plasma did not vary significantly during the experimental period in any of these human studies.

The report by Becker and Broome (1) that the selective interference by L-asparaginase of the 1st mitotic wave after partial hepatectomy of a rat is blocked by L-asparagine suggested a measure of plasma L-asparagine of rats through their postoperative course after heptectomy. At 24 and 48 hr after surgery, L-asparagine levels rose to a maximum (104 nmoles/ml) that was approximately twice the level in sham-operated control animals (64 nmoles/ml).

To determine whether L-asparagine levels in plasma were controlled by the pituitary gland, 6 male hypophysectomized rats (Charles River) were maintained postoperatively for 1 month on unsupplemented laboratory chow; normal controls were similarly maintained. The rats were exsanguinated from the abdominal aorta and levels of L-asparagine in erythrocytes and plasma were determined. In the controls, the average concentration was 52 nmoles/ml in the plasma and 90 nmoles/ml in the erythrocytes. The average L-asparagine content of the plasma and erythrocytes of the hypophysectomized group was 57 and 91 nmoles/ml, respectively. Thus, the ablation of the dominant endocrine gland, under these circumstances, failed to alter the levels of L-asparagine in blood.

Effect of Hemodialysis in Human Subjects. In an attempt to lower the plasma level of L-asparagine in human subjects without injection of a foreign protein (i.e., L-asparaginase), the potential of dialysis techniques was explored. Eight human subjects in renal failure, who were being maintained by hemodialysis twice weekly with a Travenol twin coil apparatus, were used with the above system. During the routine course of their hemodialysis, samples of dialysate and plasma were assayed for L-asparagine. The average flow of blood through the system was 260 ml/min, and the dialysate flow ranged from 200 to 400 ml/min. Chart 4 presents a summary of these studies. It can be calculated that 33% of the L-asparagine in the plasma was removed on each pass through the dialysis coils. Despite the removal of approximately 1,000,000 nmoles L-asparagine, only transient effects on the plasma concentration of this amino acid were achieved. Since approximately 10 times the total amount of L-asparagine in the plasma is removed during a 6-hr period,
extensive input from tissue stores or synthesis de novo must occur.

Biochemical Studies on a Canine Lymphoma Treated with L-Asparaginase. A 9-year-old female dachshund with spontaneous anaplastic lymphosarcoma was used as a model for subsequent studies in patients. Old et al. (17, 18) had previously shown that this type of tumor in dogs responds to treatment with L-asparaginase. After obtaining base line values, daily therapy was initiated with 10 i.u. L-asparaginase/kg i.v. As indicated in Chart 5, there was a prompt and extensive reduction in measurable tumor volume, associated with a return of the peripheral leukocyte population to essentially normal size and distribution, as well as marked clinical improvement. Associated with this improvement in the disease was the anticipated dramatic reduction in the concentration of L-asparagine in the plasma, with an associated increase in the concentration of L-aspartic acid. Biopsy of tumor nodules before and shortly after initiation of therapy revealed a sharp reduction, but not an elimination, of L-asparagine. Similarly, considerable amounts of

![Chart 4. The effect of hemodialysis on the plasma concentration of L-asparagine. Travenol twin coils were used for hemodialysis. Plasma was separated from arterial blood. The concentration of L-asparagine in the dialysate was measured spectrophotometrically. Mean blood flow rate, 260 ml/min; mean dialysate flow, 300 ml/min.](image)

![Chart 5. L-Asparaginase (E.C.-2) treatment of canine lymphosarcoma. Tumor volumes were calculated as cylinders on the basis of measurements made in 2 axes. Plasma and erythrocyte L-asparagine, L-aspartic acid, and L-asparaginase were measured spectrophotometrically.](image)
L-asparagine could be detected in erythrocytes for many days after the initiation of treatment, despite the fact that the level in the plasma was almost undetectable. The excretion of this amino acid in the urine also continued to be significant during the course of therapy with L-asparaginase. After 3 weeks of therapy at a reduced dosage, it became apparent that regrowth of the tumor was occurring. When, after 38 days, therapy was discontinued, there followed a rapid decline in the elevated concentration of L-aspartic acid in the plasma and return of the level of L-asparagine in the erythrocytes to normal levels. The plasma concentration of L-asparaginase, however, did not significantly increase for 8 days, and then returned to normal only slowly. Plasma levels of the enzyme on a fixed dose remained essentially constant throughout therapy and the determination of the half-life of the enzyme before (t½, 24 hr) and on the last day (t½, 18 hr) of therapy would suggest that immunological factors did not markedly accelerate the disappearance of enzyme activity. Thirty days after cessation of therapy, the net tumor mass had regrown to approximately the same volume as before the initial course of therapy. Daily i.v. administration of 80 i.u. L-asparaginase/kg produced a 2nd rapid regression of tumor mass and chemical changes similar to those seen during the 1st course of therapy. Unfortunately, the animal died from an intercurrent streptococcal meningitis on the 77th day.

DISCUSSION

The method presented increases both the sensitivity and the speed with which samples can be assayed for L-asparaginase. Previous methods that employed the enzymic release of ammonia have had limited sensitivity or have lacked the necessary specificity (20, 22). Automated amino acid analyzers, although effective for these measurements, have a very limited capability for multiple samples (21). Formation of the trinitrophenol derivative of L-asparagine and subsequent paper chromatography as described by Broome (4) has provided a sensitive system, but it is not easily adaptable to the processing of many samples. The enzymic method presented does have several limitations, however. Since the best procedure for preparing samples for the method reported in this paper involves heat denaturation of enzymes which might oxidize NADH, there is always the attendant problem of hydrolysis of L-asparaginase by L-asparaginase that may be present in samples obtained after therapy with this enzyme or in liver samples because of endogenous L-asparaginase activity. This is minimized by very rapid heating; but since L-asparaginase is relatively resistant to both heat and perchloric acid, the figures obtained in such an assay must be considered minimal levels. If a compound could be found which would reversibly inhibit L-asparaginase this problem would be obviated.

A comparison between the values obtained with this method and those of previous workers is somewhat difficult, since a wide range of values are presented in the literature (2, 4, 5, 21, 22). General agreement has been obtained with several of the recent papers in which the levels in plasma and experimental tumors have been measured by an amino acid analyzer (14) or the trinitrophenol method (4). The demonstrated specificity of the method and the effective recovery of added standards give further confidence in its reliability.

It was remarkable to find that despite a wide variety of advanced diseases in humans, there was little departure from the normal range of plasma concentration. These results suggest that there is very effective homeostatic control of the plasma concentration of L-asparagine, but the mechanism of this control remains to be established. The experiments with ingestion of L-asparagine confirm and extend the observations of Breuer et al. (2) and Tower et al. (22) on the ability of this amino acid to be absorbed intact from the gastrointestinal tract; furthermore, they indicate that urinary excretion plays a relatively minor role in the reestablishment of normal levels after the gross elevation in plasma concentration is achieved. Perhaps the most remarkable observation in this experiment was the slow and modest increase in the concentration of L-asparagine in the erythrocytes under these conditions.

A further indication of the metabolic controls in plasma L-asparagine was seen in the experiments with hemodialysis. The negligible effect of this procedure on plasma concentration of L-asparagine suggests that the half-time of this amino acid is less than 40 min under these conditions. Furthermore, the maximal theoretical rate of removal of L-asparagine at this considerable rate of arteriovenous shunting would be 3 times that observed if total clearance of the plasma occurred in each pass through the machine. From the experimental method it is not possible to distinguish between increased synthesis or redistribution from pre-existent stores. It seems improbable, however, that tissue L-asparagine could provide for such an extensive repletion without additional synthesis, since erythrocyte levels also were unchanged. Any method designed to reduce plasma concentrations of L-asparagine as a form of therapy must recognize that the nutritional studies in culture with L-asparagine-dependent tumor lines indicate the concentration for one-half maximal growth is approximately \(1 \times 10^5\) M (9, 17). Thus, a decrease in the plasma concentration of L-asparagine to 20% of control levels would still permit considerable tumor growth. Obviously, assuring effective killing of leukemic or other tumor cells would require a much more drastic reduction.

Evidence for the ability of tissues to sustain their endogenous pools of L-asparagine in the face of negligible levels in the plasma is seen in the experiment with the dog bearing a lymphosarcoma. The maintenance of erythrocyte and urinary concentrations of L-asparagine for many days despite its virtual absence in plasma suggests that other tissues are acting as donors to the erythrocytes which appear to have no ability to synthesize L-asparagine (D. Chou and R. E. Handschumacher, unpublished results). Similarly, the rapid reappearance of normal levels of L-asparagine in erythrocytes

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5 Recently, it has been found that in some patients with advanced lymphocytic leukemia the plasma concentration of L-asparagine is significantly below the normal range (6, 16).
after cessation of therapy must be contrasted to the very slow normalization of plasma levels, which required several days. These results are in agreement with the observation of Broome (5) concerning the slow reappearance of L-asparagine in the plasma of mice after therapy with L-asparaginase.

The data obtained in these experimental systems have considerable relevance to therapy of patients with lymphocytic and other neoplasms. A point of particular concern in clinical studies with L-asparaginase is the selection of pre-existing cells in the tumor population that are able to synthesize L-asparagine and thus are unlikely to be susceptible to the effect of this enzyme. Another possible cause for refractoriness to therapy is suggested by the current experiments. L-Asparaginase can be expected to clear the plasma of L-asparagine and reduce the concentration of this amino acid in other body fluids. It is less likely, however, that it will effectively reduce the levels of L-asparagine within cells, since the enzyme is presumably confined to the vascular space. Those cells from a disseminated disease like leukemia, which are in close proximity to normal cells capable of excreting L-asparagine, may derive sufficient amounts of this amino acid to sustain growth, even though the plasma concentration is negligible. To cope with this circumstance, a means to reduce the production of L-asparaginase in host cells is required, so that only their endogenous requirements are met and no surplus is available for excretion and the feeding of tumor cells that require exogenous L-asparagine. Although the L-glutamine analog 5-diazo-4-oxo-L-norleucine is able to block the biosynthesis of L-asparagine effectively, its other sites of inhibition limit its usefulness for this purpose. A series of other agents have been examined for this purpose in a preliminary manner in this laboratory and offer promise of more specific inhibition of the biosynthesis of L-asparagine.

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REFERENCES


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