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DON, CONV AND DONV—I. INHIBITION OF L-ASPARAGINE SYNTHETASE *IN VITRO*

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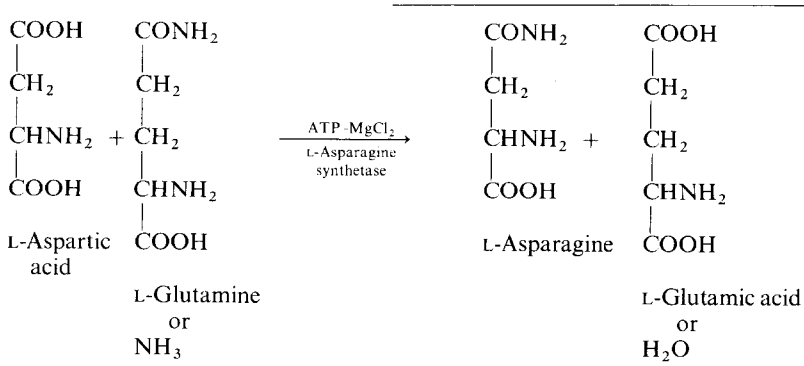
Abstract— The inhibition *in vitro* of L-asparagine synthetase (L-glutamine hydrolyzing, EC 6.3.5.4) from leukemia 5178Y rendered resistant to L-asparaginase (L5178Y/AR), and from mouse pancreas by the ketoamino acids DON (L-DON; 6-diazo-5-oxo-L-norleucine), CONV (L-CONV; 2-amino-5-chlorolevulinic acid; 5-chloro-4-oxo-L-norvaline) and DONV (L-DONV; 5-diazo-4-oxo-L-norvaline) was investigated using both L-glutamine and ammonium chloride as substrates. At a concentration of 1 mM, DON and CONV almost completely inhibited the utilization *in vitro* of L-glutamine by L-asparagine synthetase of L5178Y/AR and of mouse pancreas, whereas DONV inhibited both enzymes only by 50 per cent. DON, CONV and DONV did not affect the utilization *in vitro* of ammonium chloride by L-asparagine synthetase of L5178Y/AR, while DON and CONV modestly inhibited the utilization of ammonium chloride by the pancreatic enzyme. The inhibition produced by DONV was fully and rapidly reversed by dialysis, whereas that produced by DON and CONV was essentially irreversible. The utilization *in vitro* of L-glutamine by eight fetal rat liver amidotransferases was strongly inhibited by DON and CONV, while DONV exerted modest inhibition on only two of these enzymes. In a survey of other enzymes which use L-asparagine and L-glutamine as substrates, DONV was shown to be the best inhibitor of L-asparagine-utilizing enzymes, DON was the best inhibitor of enzymes utilizing L-glutamine, and CONV affected both groups of enzymes to a variable degree. DON irreversibly inhibited L-asparaginase from *Erwinia carotovora* (EC 3.5.1.1). This enzyme also was found to catalyze the decomposition of DON. From these findings and also from kinetic studies, it is clear that all three ketoamino acids are capable of behaving as antagonists of L-glutamine *in vitro*. DON is the most universally active antagonist of this amino acid, followed by CONV, and last by DONV. Similarly, all three agents can function as L-asparagine antagonists under appropriate conditions.

Among the antagonists of L-glutamine, only azaserine and 6-diazo-5-oxo-L-norleucine (DON) have undergone extensive clinical trials for the chemotherapy of cancer. Because of their lack of consistent effectiveness in human malignancies, clinical interest in these agents has declined [1]. The utility of L-asparaginase (EC 3.5.1.1) in the therapy of certain human neoplasms has revived interest in compounds affecting the metabolism of L-glutamine, inasmuch as the latter amino acid is a substrate in the synthesis of L-asparagine.

Several analogs of L-asparagine and of L-glutamine have been examined recently in the hope of interfering with the synthesis of L-asparagine by inhibiting cytoplasmic L-asparagine synthetase (L-glutamine hydrolyzing, EC 6.3.5.4), an enzyme which catalyzes the following amide-transfer reaction:



The present paper treats three such agents: DON (L-DON; 6-diazo-5-oxo-L-norleucine), CONV (L-CONV; 2-amino-5-chlorolevulinic acid; 5-chloro-4-oxo-L-norvaline) and DONV (L-DONV; 5-diazo-4-oxo-L-norvaline).

DON (Fig. 1), a close structural analog of L-glutamine, was first isolated in 1956 from the culture broths of an unidentified *Streptomyces* by Dion *et al.* [2]. Clarke *et al.* [3] subsequently demonstrated the activity of DON against Crocker sarcoma 180 in mice. Sullivan *et al.* [4] in a large cooperative study on acute leukemia in children, reported remissions using a combination of DON and 6-mercaptopurine. Further clinical trials using DON, singly and in combination with other antineoplastic agents, have been reviewed by Duvall [5] and Livingston *et al.* [1]. Extensive biochemical studies have shown that DON

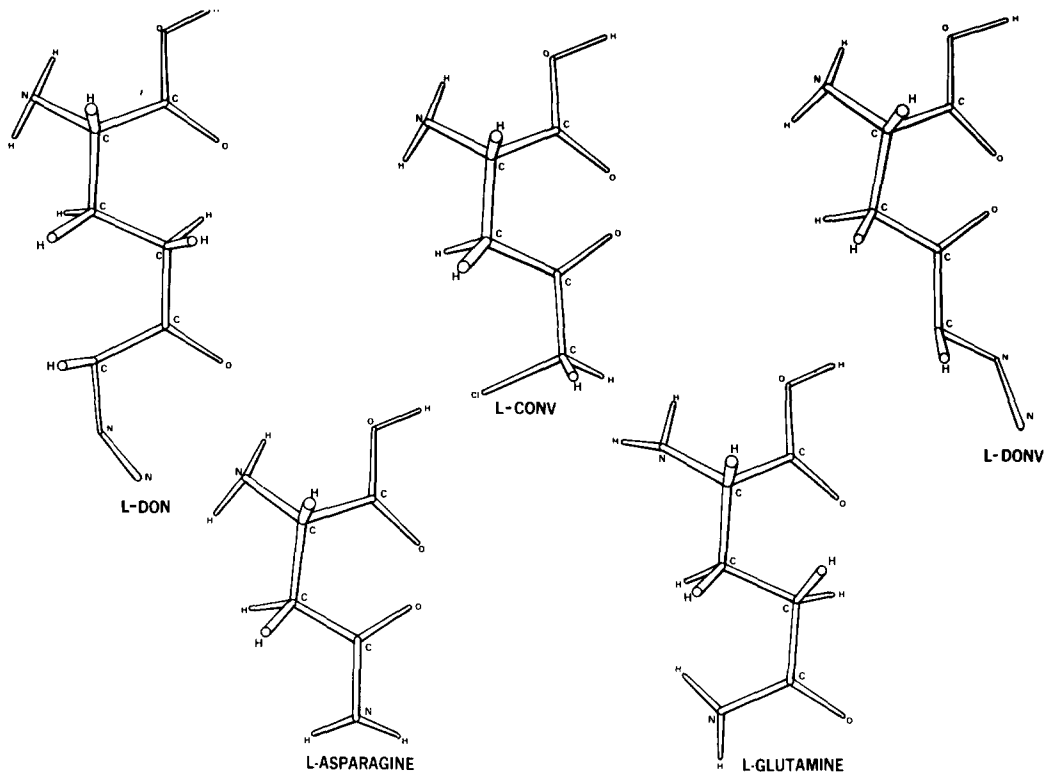


Fig. 1. Structures of DON, CONV, DONV, L-asparagine and L-glutamine.

inhibits many enzymatic reactions involving L-glutamine [6]. Of greatest significance to the present study is the finding that DON powerfully inhibits the utilization of L-glutamine by L-asparagine synthetase *in vitro* [1].

DONV (Fig. 1) was first examined by Buchanan [7] in 1958 as the next lower homologue of DON and in 1959 was proposed as an analog of L-aspartic acid by Liwschitz *et al.* [8], who demonstrated its activity in sarcoma 37 in mice. Handschumacher *et al.* [9] improved the method for the synthesis of DONV and documented its ability to inhibit L-asparaginase from *Escherichia coli*. These authors concluded that DONV was a reactive analog of L-asparagine. Summers and Handschumacher [10], working with L-asparagine-dependent and -independent clonal sublines of leukemia 5178Y, indicated that L-DONV, but not D-DONV, was capable of killing cells whose growth did not depend on an exogenous supply of L-asparagine (although an additional cytotoxic effect of both stereoisomers on both sublines could also be demonstrated). Chou and Handschumacher [11] reported inhibition by DONV of L-asparagine biosynthesis in P815 mast cell leukemia, while no significant inhibition of L-aspartic acid biosynthesis was observed.

CONV (Fig. 1), a chloroketone analog of DONV in which a halogen replaces the diazo function, was first synthesized by Miyake [12] in 1960 as an antitubercular agent. Khedouri *et al.* [13] also prepared CONV and reported that the drug inhibited the utilization of L-glutamine by carbamyl phosphate synthetase (EC 2.7.2.5). CONV has been characterized as an effective inhibitor *in vitro* of the utilization of L-glutamine by L-asparagine synthetase of Gardner lymphosarcoma and RADA1 leukemia [14]. Chou and Handschumacher [11] also established that this chloroketone impeded the biosynthesis and exportation of L-asparagine by short-term explants of the P815 leukemia. It is the purpose of the present paper to compare the biochemical properties of these three congeneric ketoamino acids, insofar as possible, under the same experimental conditions *in vitro*.

MATERIALS AND METHODS

Chemicals. DON, CONV and DONV were provided by Dr. Harry B. Wood, Jr., Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Md. Purity of the inhibitors was assessed by using the amino acid analyzer, u.v. absorption and high voltage electrophoresis. No detectable impurities were found. L[4-^{14}C]asparagine (sp. act. 12.9 mCi/m-mole) and L[U-^{14}C]asparagine (sp. act. 151 mCi/m-mole) were purchased from Amersham/Searle Corp., Arlington Heights, Il. L[U-^{14}C]-glutamine (sp. act. 220 mCi/m-mole), L[4-^{14}C]aspartic acid (sp. act. 22.2 mCi/m-mole) and L[1-^{14}C]glutamic acid (sp. act. 25 mCi/m-mole) were purchased from Biochemical Nuclear Corp., Burbank, Calif., and used without further purification. L-Glutamate oxaloacetate transaminase (GOT) (EC 2.5.1.1, sp. act. 180 I.U./mg of protein), L-glutamate pyruvate transaminase (GPT) (EC 2.5.1.2, sp. act. 80 I.U./mg of protein), and malate dehydrogenase (MDH) (EC 1.1.1.37, sp. act. 1100 I.U./mg of protein) were purchased from

Boehringer, New York, N.Y. L-Glutamate decarboxylase (EC 4.1.1.15, sp. act. 50 I.U./mg of protein) was partially purified from *E. coli* by the methods of Shukuya and Schwert [15]. L-Aspartate-β-decarboxylase (EC 4.1.1.12, sp. act. 77 I.U./mg of protein) was a generous gift of Drs. Suresh Tate and Alton Meister of the Cornell University Medical College, New York, N.Y. L-Asparaginase from *E. coli* (EC 3.5.1.1, sp. act. 340 I.U./mg of protein) was a donation to the National Cancer Institute from the Merck Institute for Therapeutic Research, Rahway, N.J. L-Asparaginase from *Erwinia carotovora* (sp. act. 500 I.U./mg of protein) was provided by the Department of Health and Social Security of Great Britain through Dr. H. E. Wade of the Microbiological Research Establishment, Porton Down, Salisbury, Wilts (SP4 0JG) England. L-Asparaginase from the serum of *Dasyprocta aguti* (sp. act. 0.16 I.U./mg of protein) was provided by Dr. F. White, Drug Evaluation Branch, National Cancer Institute, National Institutes of Health, Bethesda, Md. L-Asparaginase from the serum of *Saimuri sciurea* (sp. act. 0.03 I.U./mg of protein) was a gift of Dr. R. Morrison, Charles Pfizer, Maywood, N.J. L-Glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2, sp. act. 0.6 I.U./mg of protein) was obtained from Worthington Biochemical Corp., Freehold, N.J. All other chemicals used were of analytical reagent grade. Eppendorf 1.5-ml plastic centrifuge vessels, pipettes, pipette tips, centrifuges and incubation ovens were purchased from Brinkmann Instruments, Inc., Silver Spring, Md.

Preparation of partially purified soluble L-*asparagine synthetase.* Male BDF_1 mice, weighing between 18 and 20 g, were injected by the subcutaneous route with a suspension of 10^6 cells of leukemia 5178Y made resistant to L-asparaginase (L5178Y/AR) by repeated subcurative doses of the enzyme. Seven to 10 days later the tumours were collected and homogenized in 9 vol. of 0.1 M Tris–HCl buffer, pH 7.6, containing 0.5 mM EDTA and 1 mM dithiothreitol. The homogenate was centrifuged in the cold at 100,000 *g* in a Beckman L2-65B ultracentrifuge for 30 min and the supernatant (sp. act. 0.001 I.U./mg of protein) was collected. A portion of this supernatant was used as a source of crude L-asparagine synthetase. The remainder was partially purified (6-fold) by heating at 50° for 10 min after treating with ATP, $MgCl_2$, L-glutamine and ammonium chloride each at a concentration of 0.01 M [14]. The heated extract was immediately chilled on ice and centrifuged at 20,000 *g* for 30 min to remove the precipitated proteins and nucleic acids. The supernatant was brought to 30% saturation with solid ammonium sulfate and then centrifuged at 18,000 *g*. The precipitate was discarded and the supernatant brought to 50% saturation with solid ammonium sulfate. The resulting precipitate was suspended in 5 mM Tris–HCl buffer, pH 7.6, containing 1 mM dithiothreitol, 0.5 mM EDTA and 10% glycerol, and was dialyzed against three changes of the same buffer for 3 hr at 4°. This dialyzed extract (sp. act. 0.0058 I.U./mg of protein) was used as the source of partially purified L-asparagine synthetase of tumoral origin for inhibition studies *in vitro*.

For the preparation of L-asparagine synthetase of mouse pancreas, male BDF_1 mice, weighing between 18 and 20 g, were killed by neck dislocation, and the pancreases collected and homogenized in 9 vol. of 0.1 M Tris–HCl buffer, pH 7.6, containing 0.5 mM EDTA and 1 mM dithiothreitol. The homogenate was centrifuged in the cold at 12,000 *g* for 20 min. As before, a portion of this supernatant was used as the source of crude pancreatic L-asparagine synthetase. The remainder was partially purified (3-fold) as above. The resultant dialyzed enzyme (sp. act. 0.002 I.U./mg of protein) was used immediately as the source of pancreatic L-asparagine synthetase for inhibition studies *in vitro*.*

L-*Asparagine synthetase assay.* L-Asparagine synthetase activity was measured *in vitro* by a radiometric technique [16]. In this assay, L[4-^{14}C]aspartic acid was used as the precursor of the carbon skeleton of L-asparagine; L-glutamine or NH_4Cl served as the source of the amide, ATP–$MgCl_2$ as the energy source, and Tris–HCl, pH 8.4, as the buffer. In a final volume of 10 μl were admixed 5.6 nmoles (0.125 μCi) L[4-^{14}C]aspartic acid,† 0.2 μmole L-glutamine or 0.5 μmole NH_4Cl, 0.1 μmole ATP, 0.25 μmole $MgCl_2$, and 2.5 μmoles Tris–HCl buffer, pH 8.4, along with 5 μl of the enzyme to be assayed. After 30 min of incubation at 37° (during which time the synthesis of L[4-^{14}C]asparagine was found to be linear), the vessels were heated at 95° for 5 min to terminate the reaction, then centrifuged at 12,000 *g* for 3 min to precipitate denatured proteins. In order to remove unreacted vestiges of L[4-^{14}C]aspartic acid, an enzymatic reagent consisting of 50 μl of 0.66 M sodium acetate buffer, pH 5.0, containing 6 mM α-ketoglutaric acid, 8.5 mM $ZnSO_4$, and 1 I.U. of GOT was added to the reaction vessels and the open vessels were incubated at 37° for 4 hr. As a consequence, residual unreacted L[4-^{14}C]aspartic acid was transaminated to [4-^{14}C]oxaloacetate by GOT, which was, in turn, β-decarboxylated by the Zn^{2+} in the reagent mixture, and the resultant [^{14}C]O_2 driven from the open vessels by the acidity of the buffer. At this point, 2 I.U. of *E. coli* L-asparaginase was added to each vessel, a droplet of 40% (w/v) KOH was deposited on the underside of the lid, and the vessels were closed. In this way, any newly synthesized L[4-^{14}C]asparagine was hydrolyzed to L-[4-^{14}C]aspartic acid, which, in turn, was transmitted and ultimately β-decarboxylated, as described above. The [^{14}C]O_2 so generated was trapped by the KOH droplet [17], and after 3 hr at 37°, the lids were removed and immersed in a scintillation mixture of ethanol–toluene (1:2, v/v) containing Liquifluor (New England Nuclear, Boston, Mass.), and the radioactivity was counted in a Beckman model LS-230 liquid scintillation spectrometer at 87 per cent efficiency. Suitable blanks and standards were included in each assay to verify that the product synthesized was L-asparagine. Appropriate legends describe modifications to this basic procedure.

Attempts to prevent or reverse the inhibition of L-*asparagine synthetase.* Attempts were made to deter-

* Subsequent experience with the heating step at 50 has shown that this procedure produces a very labile enzyme with little or no purification. This method, therefore, cannot be recommended for routine use.

† The L-aspartic acid concentration (0.00056 M) used in these experiments was, for reasons of economy, substantially lower than that required to saturate the enzyme.

mine if the ordinary substrates of L-asparagine synthetase could prevent or reverse the inhibition produced by DON, CONV or DONV. For this purpose, inhibitor was added to 50 μl of crude tumoral L-asparagine synthetase to a final concentration of 1 mM (in the case of DON and CONV) or 5 mM (in the case of DONV) either 15 min before (for reversal studies) or 15 min after (for prevention studies) L-glutamine (0.01 to 0.1 M), NH_4Cl (0.1 M), ATP–$MgCl_2$ (0.01 M), L-aspartic acid (0.5 to 5 mM) or dithiothreitol (0.05 M). After 30 min at 25°, the enzyme was diluted 5-fold with 0.01 M Tris–HCl buffer, pH 8.4, and assayed for L-asparagine synthetase activity in the usual manner.

To investigate the possibility of reversal of the inhibition exerted by DON, CONV and DONV by dialysis, crude extracts of L5178Y/AR were exposed to concentrations (10–20 mM) of the drugs calculated to inhibit the utilization of L-glutamine by L-asparagine synthetase by approximately 80–90 per cent, and then dialyzed exhaustively against 0.1 M Tris–HCl buffer, pH 7.6, in the presence or absence of 1 mM dithiothreitol. The retentate then was assayed for L-asparagine synthetase activity as described above.

Assay of amidotransferases utilizing L-glutamine. The effect of the inhibitors on some of the other L-glutamine-utilizing amidotransferases was examined in fetal rat liver. For this purpose, livers from twenty midterm fetuses were homogenized in 0.1 M Tris–HCl buffer, pH 7.6, containing 0.5 mM EDTA and 0.1 mM dithiothreitol. After centrifugation at 105,000 *g* for 30 min, the supernatant was dialyzed for 6 hr against three changes of 1 liter each of homogenizing buffer containing 20% (v/v) glycerin. This step was necessary to remove endogenous substrates. The assay measures the substrate-dependent breakdown of L-[U-^{14}C]glutamine to L[U-^{14}C]glutamic acid. In a final volume of 25 μl were admixed 1 μCi L[U-^{14}C]glutamine, neutralized substrates (cf. Table 4) to a final molarity of 0.01 M, 0.05 M Tris–HCl buffer, pH 7.6, ATP–$MgCl_2$ to a final molarity of 0.01 M, and to initiate the reaction, 10 μl of dialyzed liver extract. Two sets of blanks were constituted: the first received all ingredients except the substrate (amide recipient), while the second received all ingredients except ATP–$MgCl_2$. The first set of blanks allowed an estimation of the basal L-glutaminase activity of the extract, while the second set, an estimate of ATP-independent amide transfer. After incubation at 37° for 30 min, the reaction was terminated by heating at 95° for 5 min. Any L[U-^{14}C]glutamic acid produced in the incubation step was subsequently quantitated by the addition of 50 μl of 0.2 M sodium acetate buffer, pH 4.2, containing 1 I.U. of L-glutamate decarboxylase. The [^{14}C]O_2 resulting from this step was collected in droplets of 40% (w/v) KOH deposited on the underside of the lid of the reaction vessel [17]. Under the conditions of this assay, DON, CONV and DONV did not interfere with the quantitative decarboxylation of L-glutamate.

L-Glutamine synthetase assay. L-Glutamine synthetase activity (EC 6.3.1.2) was measured *in vitro* by a radiometric technique. The reaction allows the synthesis of L[1-^{14}C]glutamine from L[1-^{14}C]glutamic acid in the presence of $MgCl_2$, ATP and NH_4Cl. Brains and livers were removed from male BDF_1 mice and homogenized as described above. Five μl of the 1:10 (w/v) tissue homogenate was incubated at 37° for 10 min with 5 μl of a reaction mixture containing 0.02 M ATP–$MgCl_2$, 0.04 M L-glutamic acid, 0.1 μCi L[1-^{14}C]glutamic acid, 0.16 M NH_4Cl, in the presence or absence of DON, CONV and DONV at a final concentration of 1.0 mM. The reaction was terminated by heating the vessels at 95° for 3 min. The vessels were centrifuged at 12,000 *g* for 3 min to precipitate any denatured protein. Unreacted L[1-^{14}C]glutamic acid was removed by the addition of 1 I.U. of L-glutamate decarboxylase in 0.33 M sodium acetate buffer, pH 4.2, followed by incubation for 2 hr at 37° [18]. L[1-^{14}C]glutamine then was hydrolyzed to L[1-^{14}C]glutamate by the addition of 20 I.U. of L-asparaginase. The [^{14}C]O_2 generated by the subsequent α-decarboxylation by L-glutamate decarboxylase was collected and counted as described in the assay procedure for L-asparagine synthetase.

Measurement of transaminases. L-Asparagine transaminase (EC 2.6.1.14) activity and L-glutamine transaminase (EC 2.6.1.15) activity were measured radiometrically. The enzyme was a 12,000 *g* supernatant of a 1:5 (w/v) homogenate of BDF_1 mouse liver; 0.05 M Tris–HCl buffer, pH 7.4, rendered 0.5 mM in EDTA and 1 mM in dithiothreitol, was used for the homogenization. After centrifugation, the supernatant was dialyzed overnight at 4° against 4 liters of the homogenization buffer containing 20% (v/v) glycerol and used immediately. The assay measures the conversion of the radioactive amides to a product α-decarboxylated by acid hydrogen peroxide. In Eppendorf vessels, in a final volume of 15 μl were admixed: 5 μl (0.25 μCi) L[U-^{14}C]asparagine or L[U-^{14}C]glutamine, 5 μl of 0.03 M α-ketovaleric acid in 0.05 M Tris–HCl buffer, pH 8.4, or 5 μl of the buffer alone (as a control) and 5 μl of crude transaminase. After 30 min at 37°, 50 μl 1 M HCl rendered 1% (v/v) in H_2O_2 was added to the reaction mixture at the same time as a 5-μl droplet of 40% (w/v) KOH was deposited on the underside of the lid of the vessel. One hr later the radioactivity on the lids was measured by scintillation spectrometry.

Measurement of L-asparaginase and L-glutaminase. L-Asparagine activity was measured by a spectrophotometric technique [19] in which 0.01 M L-asparagine was used as substrate. L-Glutaminase activity was measured by a radiometric technique: in a final volume of 20 μl were admixed 0.25 μCi, 9 nmoles, L[U-^{14}C]glutamine, 5 μl of 0.05 M Tris–HCl buffer, pH 8.4, or inhibitor at a concentration of 4 mM in the same buffer, 5 μl of 0.4 M sodium acetate buffer, pH 5.0, and to initiate the reaction, 5 μl of crude L-glutaminase. After incubating at 37° for 30 min, the vessels were heated at 95° for 3 min. L[U-^{14}C]glutamic acid was measured by means of L-glutamate decarboxylase as described earlier.

RESULTS

Inhibition of L-asparagine synthetase. A comparison of the relative inhibitory potencies of DON, CONV and DONV against partially purified soluble L-asparagine synthetase from L5178Y/AR and from mouse pancreas is presented in Table 1. DON and CONV were potent inhibitors of tumoral as well as pancrea-

Table 1. Inhibition *in vitro* of partially purified soluble L-asparagine synthetase of L5178Y/AR and of mouse pancreas, using L-glutamine and ammonia as substrates*

Inhibitor	Concn (mM)	Per cent inhibition of L-asparagine synthetase from: L5178Y/AR		Mouse pancreas	
		L-Glutamine	Ammonia	L-Glutamine	Ammonia
DON	10.0	94	0	95	25
	1.0	78	0	86	17
	0.1	30	0	43	13
CONV	10.0	91	0	95	46
	1.0	87	0	89	43
	0.1	53	0	39	39
DONV	10.0	49	4	62	7
	1.0	36	0	43	0
	0.1	16	0	24	0

* The reaction mixture consisted in a final volume of 45 μl. 3 nmoles (0.07 μCi) L[U-^{14}C]aspartic acid. 1 μmole L-glutamine or 2.5 μmoles NH_4Cl, 1.5 μmoles ATP and $MgCl_2$, and 2.5 μmoles Tris–HCl buffer, pH 8.4, along with 5 μl of crude enzyme from tumor or pancreas. Procedures for the incubation are described in Materials and Methods. The specific activities of the uninhibited tumoral and pancreatic enzymes were approximately 40 and 25 nmoles/mg of protein/hr respectively.

tic L-asparagine synthetases when L-glutamine was used as the amide donor. In this system, however, DONV was decidedly less effective at each concentration as an inhibitor of L-glutamine utilization by both enzymes. From Table 1, it can also be appreciated that DON, CONV and DONV did not inhibit the tumoral L-asparagine synthetase when ammonia (as NH_4Cl) was the source of the amide, although modest inhibition of ammonia utilization by the pancreatic L-asparagine synthetase was observed with CONV, and to a lesser extent, with DON.* In experiments not included in Table 1, a similar inhibitory pattern was observed with crude preparations of L-asparagine synthetase from tumor and pancreas.

Characteristics of the inhibition of L-asparagine synthetase by DON, CONV and DONV. To delineate more precisely the characteristics of the inhibition of tumoral L-asparagine synthetase by each of the three drugs under investigation, specific kinetic parameters were examined. To minimize irreversible inhibition of the enzyme (see below), 5-min incubations were used. At low L-glutamine concentrations (0.625 to 5.0 mM), DONV and CONV appeared to act as noncompetitive inhibitors versus this substrate; when the L-glutamine concentration was elevated above 5 mM, however, these agents exerted inhibition which was formally competitive in type (Fig. 2). By contrast, DONV produced apparently competitive inhibition at all concentrations of L-glutamine. Not shown is the additional observation that DONV (the only agent of the three examined with L-aspartic acid as the variable substrate) produced noncompetitive inhibition in this case.

Reversal of inhibition. Having determined that all three of these ketoamino acids could inhibit L-asparagine synthetase, it became important, for pharmacologic reasons, to ascertain whether the inhibition was reversible by dialysis. Crude extracts of L5178Y/AR were exposed to concentrations (10–20 mM) of the drugs calculated to produce approximately 80–90 per cent inhibition of the utilization of L-glutamine by L-asparagine synthetase, and then dialyzed exhaustively at 4 against 0.05 M Tris–HCl buffer, pH 7.6, in the presence or absence of 1 mM dithiothreitol. The inhibition exerted by DONV was nearly fully reversed under these conditions, whereas that exerted by CONV and DON showed only marginal reversibility (Fig. 3). Moreover, dithiothreitol in the outer dialysis bath failed to reverse significantly the inhibition produced by DON and CONV.

The ability of L-glutamine and L-aspartic acid as well as of the other substrates of L-asparagine synthetase to prevent or reverse the inhibition produced by DON, CONV and DONV was investigated next. Since these modifiers are normal constituents of body fluids, this study was expected to provide insight into the behavior of these drugs in the intact organism. Of the substrates tested, L-aspartic acid and ATP–$MgCl_2$ did not either prevent or reverse the inhibition of L-asparagine synthetase produced by DON, CONV and DONV. L-Glutamine at a concentration of 10 mM (Table 2) also failed to prevent or reverse the inhibition exerted by any of these acids, but at 100 mM did retard significantly the inhibitory action of all three. Stated otherwise, a 20- to 100-fold molar excess of L-glutamine was required to shield the enzyme partially from inhibition by DON, CONV

* While this manuscript was in press we discovered that inhibition of the utilization of NH_4Cl by L-asparagine synthetase of L5178Y/AR by DON, CONV and DONV is apparently dependent on the substrate concentrations in the reaction mixture. Thus, while no inhibition of NH_4Cl utilization by the tumoral enzyme was observed at a 1-mM concentration of DON and CONV and at a 10-mM concentration of DONV (Table 1) when the NH_4Cl, ATP, $MgCl_2$ and L-aspartic acid concentrations were 0.055, 0.033, 0.033 and 0.00066 M, respectively, 72% inhibition by DON (1 mM), 43% inhibition by CONV (1 mM) and 47% inhibition by DONV (10 mM) was produced when the concentrations of NH_4Cl, ATP, $MgCl_2$ and L-aspartic acid were lowered to 0.033, 0.007, 0.017 and 0.00056 M, respectively.

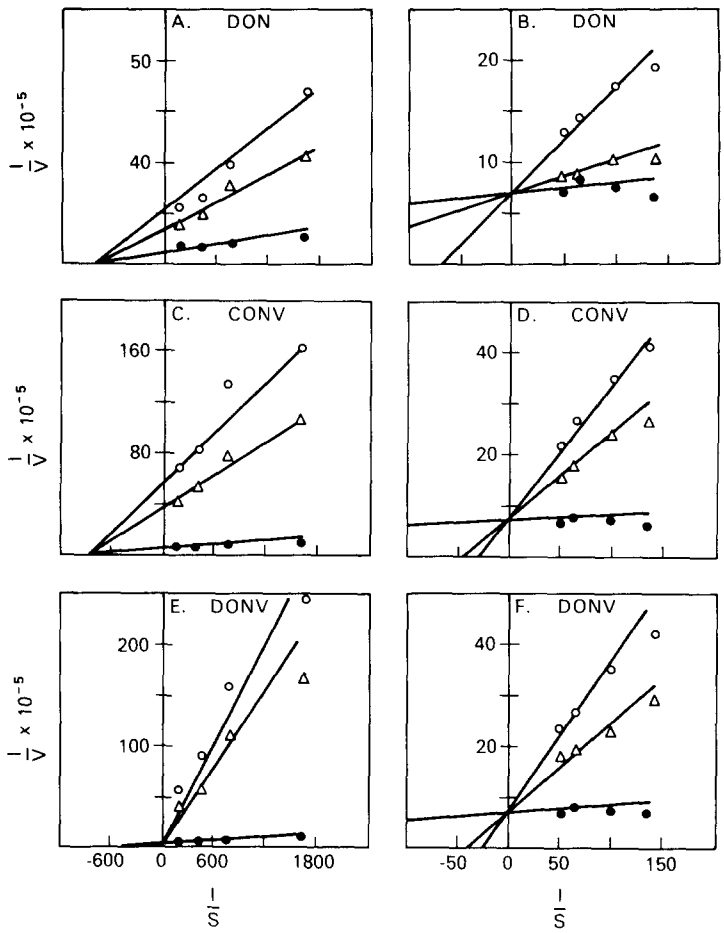


Fig. 2. Double-reciprocal analysis of the inhibition of L-asparagine synthetase of L5178Y/AR by DON, CONV and DONV vs L-glutamine. Seven and a half ml of a 30–50% $(NH_4)_2SO_4$ fraction of L-asparagine synthetase from the L5178Y/AR was prepared as described in Materials and Methods with the exception that the 50° heating step was omitted. Subsequently, the preparation was dialyzed extensively at 4° for 16 hr against two changes of 4000 ml each of 0.01 M Tris–HCl buffer, pH 7.6, containing 0.1 mM dithiothreitol and 0.1 mM disodium–EDTA. The dialyzed enzyme was concentrated to 2 ml against Carbowax 20,000 (Fisher Scientific Co., Silver Spring, Md.). The concentrated enzyme (5 μl) was assayed for L-asparagine synthetase activity in the presence of DON (1 mM = ○, 0.5 mM = △), CONV (1 mM = ○, 0.5 mM = △), DONV (10 mM = ○, 5 mM = △), or water (●) essentially as described in Materials and Methods with the exception that the incubation time was limited to 5 min at 37° and the L-glutamine concentration was varied as follows: 0.000625, 0.00125, 0.0025 and 0.005 M (panels A, C and E) and 0.0075, 0.01, 0.015 and 0.02 M (panels B, D and F). The L-aspartic acid and ATP–Mg^{2+} concentrations were 0.000475 and 0.01 M respectively. The specific activity of L-asparagine synthetase at saturation was 40 nmoles/mg of protein/hr. S = molarity; V = cpm.

and DONV, a finding which underscores the great affinity of these inhibitors for L-asparagine synthetase. On the other hand, L-aspartic acid wholly failed to antagonize inhibition exerted by these ketoamino acids. Inasmuch as the experimental design of these studies required subdilution of the inhibited enzymes prior to measurement of L-asparagine synthetase activity and inasmuch as the inhibition produced by DONV was found to be reversible, separate experiments were undertaken with this ketoamino acid in which L-aspartic acid and inhibitor were added to the assay mixture. Even in this case, L-aspartic acid was ineffective as a counteragent at 1 mM and protected only by 8 per cent at a concentration of 15 mM.

Of note was the pronounced protective effect of dithiothreitol on the inhibition exerted by all three ketoamino acids. The implications of this finding will be discussed below.

Despite the fact that the rate of utilization of ammonia by tumoral L-asparagine synthetase was comparatively refractory to inhibition by the title compounds, attempts were made to determine whether these agents could modify this activity in some subtle way not detectable under conditions of substrate saturation. To explore this point, tumoral L-asparagine synthetase was incubated with concentrations (10–20 mM) of DON, CONV and DONV known to produce 80–90 per cent inhibition of the enzyme. After incubation at 37° for 30 min, inhibitors were removed by exhaustive dialysis at 4°, and the affinity constants of surviving or restored enzyme were measured radiometrically. It is clear from Table 3 that the affinity for ammonia of tumoral L-aspara-

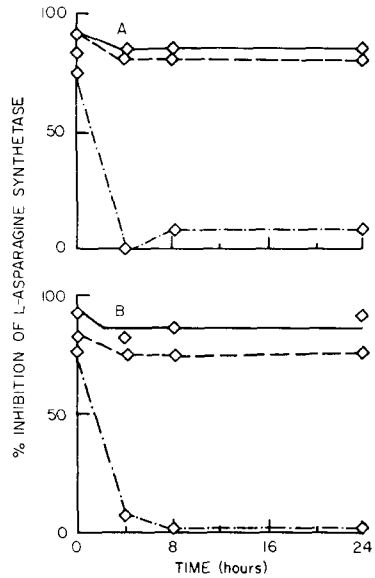


Fig. 3. Effect of dialysis on the inhibition of L-asparagine synthetase by DON, CONV and DONV. A 1:10 (w/v) homogenate of L5178Y/AR was prepared in 0.1 M Tris–HCl buffer, pH 7.6, containing 0.5 mM EDTA. Neutralized DONV (◇–●–◇) solution was added to a final concentration of 0.05 M. DON (◇–◇) and CONV (◇–◇) were added to final concentrations of 5 mM. After 30 min at 37°, the activity of L-asparagine synthetase was estimated radiometrically. The tumor extract was then dialyzed against 0.01 M Tris–HCl buffer, pH 7.6, containing 0.5 mM EDTA, 10% (v/v) glycerol either with (A) or without (B) 1 mM dithiothreitol. At the times indicated, samples were taken for radiometric analysis of L-asparagine synthetase activity using L-glutamine or NH_4Cl as substrate. In a final reaction volume of 10 μl, the concentrations of L[4-^{14}C]aspartic acid, L-glutamine (or NH_4Cl), ATP, and $MgCl_2$ were 5.6 nmoles, 0.2 μmole (or 0.5 μmole), 0.1 μmole and 0.25 μmole respectively. The specific activity of the uninhibited enzyme was 35 nmoles/mg of protein/hr. Subsequent steps in the assay are as described in Materials and Methods.

Table 3. Michaelis constants and V_{max} for ammonia of L-asparagine synthetase of L5178Y/AR inhibited with either DON, CONV or DONV and then subjected to dialysis*

Inhibitor	K_m (mM)	Relative V_{max} (%)
Buffer control	6.2	100
DON	25.0	100
CONV	25.0	100
DONV	6.2	100

* Crude L-asparagine synthetase of L5178Y/AR extract (1.0 ml) was incubated at 4° with DON, CONV or DONV (final concentration 4.5 mM) or dialyzing buffer [containing 0.1 M Tris–HCl buffer, pH 7.6, 0.5 mM EDTA, 1.0 mM dithiothreitol and 20% (v/v) glycerol] for 30 min with occasional stirring. The incubates were then dialyzed against 300 ml of dialyzing buffer for 4 hr at 4°. Dialysates were used as the source of enzyme for the study of Michaelis constants and V_{max} as described earlier.

gine synthetase treated in this way is significantly and similarly depressed (i.e. the K_m values are elevated) by DON and CONV, whereas the V_{max} is unaltered. These results indicate that the catalytic properties of the enzyme have been permanently altered by exposure to DON and CONV, irrespective of substrate.

In Fig. 4, the time course of the inhibition of partially purified tumoral L-asparagine synthetase by DON and CONV is presented, along with an examination of the protective action of a number of counteragents. It can be appreciated from this figure that CONV required only 2 min to achieve 50 per cent of the maximal inhibition ultimately exerted. After 5 min of incubation with CONV, enzyme activity was still approximately 45 per cent of control. DON, however, required over 30 min to reduce the activity of L-asparagine synthetase to 50 per cent of control in the presence of all the substrates. Also apparent is the finding that no other substrate is required for

Table 2. Effect of dithiothreitol, L-glutamine, L-aspartic acid and ATP–$MgCl_2$ on the inhibition of L-asparagine synthetase of L5178Y/AR by DON, CONV and DONV*

	Effect on inhibition of L-asparagine synthetase by:					
	DON		CONV		DONV	
Agent (concn)	Prevention (%)	Reversal (%)	Prevention (%)	Reversal (%)	Prevention (%)	Reversal (%)
Dithiothreitol						
(50 mM)	66	0	67	17	100	[38]
L-Glutamine						
(10 mM)	0	0	0	0	0	0
(100 mM)	50		58		43	13
L-Aspartic acid						
(0.5 mM)	0		0		0	
(5.0 mM)	0		0		0	
ATP–$MgCl_2$						
(10 mM)	0	0	0	0	0	0

* Protection and reversal studies were conducted according to the procedures outlined in Materials and Methods, in which enzyme activity was measured in a reaction volume of 10 μl consisting of 5.6 nmoles L[4-^{14}C]aspartic acid, 0.2 μmole L-glutamine, 0.1 μmole ATP, 0.25 μmole $MgCl_2$ and 2.5 μmoles Tris-HCl buffer, pH 8.4. Brackets indicate that the effect is probably not true reversal inasmuch as DONV apparently produces inhibition that is freely reversible on subdilution, dialysis, etc.

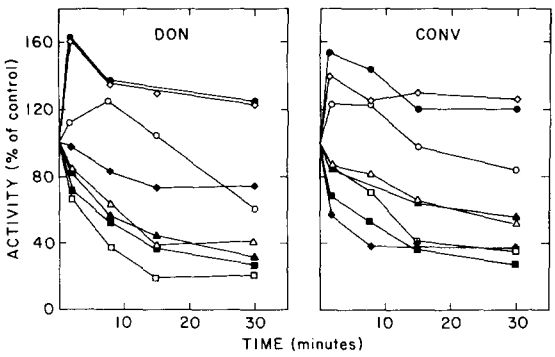


Fig. 4. Time course of the inhibition of L-asparagine synthetase by DON and CONV. Aliquots (100 μl) of a partially purified extract of L5178Y/AR dialyzed 8 hr against 0.1 M Tris–HCl buffer, pH 7.6, containing 0.5 mM EDTA and 20% (v/v) glycerin were added to nine Eppendorf 1500-μl conical vessels containing in a final volume of 125 μl either 0.125 μmole of drug (△), 0.125 μmole of drug and 10.0 μmoles of L-glutamine (○), 0.125 μmole of drug plus 0.125 μmole of L-aspartic acid (▲), 0.125 μmole of drug plus 1.25 μmoles of ATP–$MgCl_2$ (■), 0.125 μmole of drug plus 1.25 μmole of ATP–$MgCl_2$ plus 0.125 μmole of L-aspartic acid (□), 0.125 μmole of drug plus 10.0 μmoles of L-glutamine plus 0.125 μmole of L-aspartic acid plus 1.25 μmoles of ATP–$MgCl_2$ (◆), 1.25 μmoles of dithiothreitol (◇), 0.125 μmole of drug and 1.25 μmoles of dithiothreitol (●), or water and incubated at 25° for 30 min. At the times indicated, 20 μl of the incubation mixture was removed and added to 480 μl of 0.1 M Tris–HCl buffer, pH 7.6, containing 0.5 mM EDTA. The vessel was inverted and 5 μl of the resulting mixture was added to 5 μl of a radioactive mixture containing L[4-^{14}C]aspartic acid, L-glutamine, ATP, and $MgCl_2$, at 5.6 nmoles, 0.2 μmole, 0.1 μmole and 0.25 μmole respectively, and assayed for L-asparagine synthetase activity as described in Materials and Methods.

these two agents to produce inhibition. Nevertheless, ATP–$MgCl_2$ accelerated the rate and augmented the absolute degree of inhibition in both cases; L-aspartic acid did not. Furthermore, the presence of ATP–$MgCl_2$ and L-aspartic acid overcame the protective effect of L-glutamine. These findings may indicate that the inhibitors interact preferentially with the adenylated form of the enzyme [14]. The stimulatory action of dithiothreitol is also revealed in Fig. 4. Such activation has previously been reported by Chou [20] using L-asparagine synthetase from the Gardner lymphosarcoma, as well as by Horowitz and Meister [14] using the enzyme from the RADA1 leukemia. This is further evidence for the essential role played by sulfhydryl groups in the catalytic function of this class of synthetases.

Inhibition by DON, CONV and DONV of fetal rat liver amidotransferases. The demonstration of the inhibition of L-asparagine synthetase by DON, CONV and DONV prompted the study of the inhibitory potency of these agents against several additional amidotransferases which similarly utilize L-glutamine as an amide donor. All of the fetal rat liver amidotransferases examined (Table 4) were powerfully inhibited by DON and CONV; DONV, on the other hand, exhibited modest inhibition only of fetal rat liver L-asparagine synthetase and L-glutamine-D-fructose-6-phosphate amidotransferase (EC 2.6.1.16).

Inhibition of L-glutamine synthetase. In the experiments with L-asparagine synthetase presented in Table 1, it appeared that DON, CONV and, to a lesser extent, DONV, behaved as anatgonists of L-glutamine. Accordingly, the interaction of these drugs with L-glutamine synthetase from mouse brain and liver was examined. Inasmuch as L-glutamine behaves as a product inhibitor of L-glutamine synthetase [21] under some circumstances, it might be conjectured that structural analogs of L-glutamine would inhibit

Table 4. Inhibition by DON, CONV and DONV of selected amidotransferases in fetal rat liver extracts*

Substrate added	Enzyme	EC No.	Per cent inhibition of the utilization of L[U-^{14}C]glutamine in the presence of:		
			DON	CONV	DONV
None			80	62	15
L-Aspartic acid	L-Asparagine synthetase	6.3.5.4	100	86	54
Desamido-NAD	NAD synthetase	6.3.5.1	74	73	0
FGAR (5′-phosphoribosyl-formyl glycineamide)	FGAR synthetase	6.3.5.3	100	100	0
XMP (xanthosine-5′-phosphate)	XMP aminase	6.3.5.2	100	94	0
D-Fructose-6-phosphate	L-Glutamine-D-fructose-6-phosphate amido-transferase	2.6.1.16	93	96	20
PRPP (5′-phosphoribosyl-pyrophosphate)	PRPP amidotransferase	2.4.2.14	100	99	0
UTP (uridine-5′-triphosphate)	CTP synthetase	6.3.5.X	85	95	0
$NaHCO_3$	Carbamyl phosphate synthetase	2.7.2.X	55	61	6

* Inhibitors were added to a final concentration of 1 mM. The substrate-dependent hydrolysis of L-glutamine was carried out as described in Materials and Methods.

Table 5. Inhibition of L-glutamine synthetase and L-asparagine- or L-glutamine-utilizing enzymes by DON, CONV and DONV*

Enzyme	Per cent inhibition in the presence of:		
	DON	CONV	DONV
L-Glutamine synthetase (EC 6.3.1.2) from *Mus musculus* liver	0	17	10
L-Glutamine synthetase (EC 6.3.1.2) from *M. musculus* brain	0	10	2
L-Glutamine transaminase (EC 2.6.1.15) from *M. musculus* liver	17	13	30
L-Glutaminase (EC 3.5.1.2) from *E. coli*	85	0	0
L-Asparaginase (EC 3.5.1.1) from *E. carotovora*	40	46	94
L-Asparaginase (EC 3.5.1.1) from *E. coli*	5	0	69
L-Asparaginase (EC 3.5.1.1) from *D. aguti* plasma	15	8	60
L-Asparaginase (EC 3.5.1.1) from *S. sciurea* plasma	0	86	98
L-Asparagine transaminase (EC 2.6.1.14) from *M. musculus* liver	0	45	20

* For the measurement of L-asparaginase activity, suitable aliquots of a solution containing 10 I.U./ml of the enzymes from the sources listed were incubated at 37 for 30 min in 0.05 M Tris–HCl buffer, pH 8.4, containing either 1 mM L-asparagine (in the case of *E. coli* and *E. carotovora* L-asparaginase) or 10 mM L-asparagine (in the case of all other L-asparaginases). Inhibitor was added to a final concentration of 1 mM. The L-aspartic acid so generated was measured spectrophotometrically [19]. The activities of the transaminases, L-glutamine synthetase and L-glutaminase were assayed radiometrically as described in Materials and Methods.

this enzyme. However, none of the agents produced significant inhibition of L-glutamine synthetase from mouse brain and liver (Table 5).

Inhibition of L-asparagine- and L-glutamine-utilizing enzymes by DON, CONV and DONV. The interaction of these three inhibitors with a panel of additional enzymes acting on, or generating four or five carbon dicarboxylic amino acid amides, was examined next. The results of this study, presented in Table 5, indicated that DONV was the most general and potent of the inhibitors of the L-asparagine amidohydrolases examined: the activity of both the bacterial and mammalian L-asparaginases was significantly reduced by this diazoketone, at a concentration of 1 mM.

While DON and CONV both failed to inhibit L-asparaginase from *E. coli*, it is worthwhile stressing that the analogous hydrolase from the plant pathogen, *E. carotovora*, was significantly inhibited by both agents. This inhibition by DON was studied further. From double-reciprocal plots, DON appeared to be competitive with L-asparagine (Fig. 5). However, that the inhibition was more complex than this was indicated by the finding that dialysis wholly failed to reverse it. This result raised the possibility that DON was alkylating the enzyme. To investigate this point, a spectrophotometric technique was used. It was reasoned that the diazonium group would very likely be lost in the course of any alkylation, giving rise to gaseous N_2 and an enzyme with 6-hydroxy-5-oxo-L-norleucine covalently coupled to some functional group at its active site. Since the strong ultraviolet absorbance of DON is a consequence of its diazonium group, it follows that a decline in absorbance at the λ_{max}, 274 nm, would be observed with alkylation. If alkylation is the sole explanation for this effect it also follows that the decline should be stoichiometric with the number of active sites on the mol-

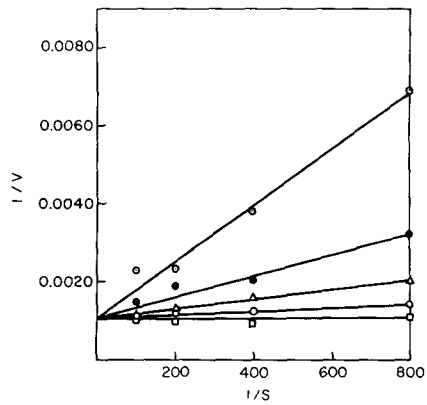


Fig. 5. Inhibition of L-asparaginase from *E. carotovora* by DON; double-reciprocal analysis. L-asparaginase (0.061 I.U.) from *E. carotovora* was incubated at 37° for 30 min with 200 µl of 0.01 M L-asparagine in 0.05 M Tris–HCl buffer, pH 8.4, with DON at 1 mM (○), 2 mM (△), 4 mM (●) and 8 mM (⊙) or without DON (□). The reactions were terminated by the addition of 50 µl of 2 M HCl followed in 10 min by 50 µl of 2 M NaOH. Any L-aspartic acid produced was measured spectrophotometrically as described in Materials and Methods.

ecule. Experimentally it was observed that 1 nmole L-asparaginase from *Erwinia* can decompose up to 15 nmoles DON before the enzyme is inactivated. That this effect was attributable to the amidohydrolytic activity of the L-asparaginase used, and not to some contaminant, was shown by the ability of an 80-fold molar excess of alternate substrate, D-asparagine, to inhibit the decomposition of DON by 50 per cent. These findings warrant the conclusion that DON is interacting in two ways with this amidohydrolase: (1) the diazoketone is capable of covalently alkylating the enzyme, and (2) the enzyme is capable of catalytically decomposing the diazoketone. These interactions are comparable to those of DONV with L-asparaginase from *E. coli* [22]. However, it should be pointed out that whereas the L-asparagine amidohydrolase from *E. coli* decomposes DONV rapidly, its counterpart from *Erwinia* decomposes DON at a very feeble rate.

Only DON exerted inhibition against the pH 5.0 L-glutaminase of *E. coli* (Table 5). Hartman [23–25] has published a comprehensive analysis of this interaction and has reported that the diazoketone alkylated the enzyme, giving rise to irreversible inhibition. On the other hand, DON alone of the three agents examined failed to inhibit mouse liver L-asparagine transaminase. CONV was a more powerful inhibitor of this transaminase than DONV, but neither agent exhibited outstanding potency. The same was true of all three ketoamino acids against L-glutamine transaminase of mouse liver. In this case, however, the order of inhibitory potency differed from that anticipated: DONV proved to be the best inhibitor of L-glutamine transaminase, while CONV and DON were both appreciably less active. It is of interest that DON appeared to permit transamination in the absence of an exogenous ketoacid. This finding may indicate that DON has itself been transaminated to the 2-keto form by the crude preparation used, or even that the 4-keto function adjacent to a diazonium group can be transaminated, possibly by analogy to the recognized transamination of succinic semialdehyde.

DISCUSSION

Inhibition of tumor growth by L-asparaginase is presumed to be associated with the depletion of cellular L-asparagine. The development of enhanced synthesis of L-asparagine from L-aspartic acid and L-glutamine (or NH_3) via the enzyme L-asparagine synthetase has been linked with clinical resistance to therapy with L-asparaginase [1]. A variety of animal tumors sensitive to L-asparaginase contain low levels of L-asparagine synthetase [26]; repeated treatment of animals bearing these tumors with subcurative doses of L-asparaginase results in increased levels of L-asparagine synthetase and the concomitant development of resistance to L-asparaginase [27]. It follows from this sequence that effective inhibition of L-asparagine synthetase should (1) increase the therapeutic efficacy of L-asparaginase in L-asparaginase-sensitive tumors by preventing the development of resistance, and possibly (2) convert tumors already resistant to L-asparaginase back to the sensitive state. Structural analogs of L-glutamine might be expected to compete for the L-glutamine-binding sites on L-asparagine synthetase and thus serve as effective inhibitors of this enzyme. Accordingly, a variety of these analogs have undergone trials as possible inhibitors of L-asparagine synthetase [1].

Haskell and Canellos [28] reported strong inhibition of L-glutamine utilization by L-asparagine synthetase of KB human tumor cells exposed to the ketoamino acids DON and DONV. Livingston *et al.* [1], in studying the effects of L-glutamine analogs on L-asparagine synthetase from Gardner lymphosarcoma, discovered that DON and CONV significantly inhibited the utilization of L-glutamine but not of NH_3 by the enzyme. Chou and Handschumacher [11] demonstrated that CONV and DONV were both potent inhibitors *in vitro* of L-glutamine utilization by L-asparagine synthetase of the murine mastocytoma P815, while Horowitz and Meister [14] showed that CONV inhibited the utilization of L-glutamine as well as of ammonia by the enzyme from L-asparaginase-resistant RADA1 mouse leukemia.

In the present studies *in vitro* on L-asparagine synthetase from L5178Y/AR, both DON and CONV behaved as potent inhibitors of L-glutamine utilization by the enzyme; DONV was a comparatively weak inhibitor. None of the three ketoamino acids exerted any significant effect on NH_3 utilization. As an explanation for the difference between these results and those of Horowitz and Meister (using RADA1 leukemia) [14] it may be suggested that L-asparagine synthetase from the latter neoplasm is biochemically similar to the L-asparagine synthetase of mouse pancreas, an enzyme whose utilization of NH_3 was shown to be partially inhibited by DON and CONV in the present study.

Further analysis of the inhibitory characteristics of the ketoamino acids against L-asparagine synthetase from L5178Y/AR revealed that all three agents appeared to inhibit competitively with L-glutamine as substrate. However, at L-glutamine concentrations below 5 mM, DON and CONV showed noncompetitive inhibition toward L-glutamine utilization by L-asparagine synthetase. These findings are consistent with those of Horowitz and Meister [14], who showed that CONV appears to compete with L-glutamine for utilization by RADA1 L-asparagine synthetase. However, in view of the irreversible nature of the inhibition produced by DON and CONV, the apparent patterns of inhibition seen in the kinetic experiments with these agents must be viewed with caution [29].

Nevertheless, in the case of at least three classical and irreversible antagonists of L-glutamine, unambiguous, competitive double-reciprocal plots have been published. Thus, Chu and Henderson [30] presented good diagrammatic evidence that azaserine (*O*-diazoacetyl-L-serine) inhibited phosphoribosyl formyl glycinamidine synthetase (EC 6.3.5.3) in an apparently competitive manner; Mazlen [31] and Hartman [32] documented that the early interaction of DON with L-glutamine-D-fructose-6-phosphate amidotransferase (EC 6.3.1.19) and phophoribosyl pyrophosphate amidotransferase (EC 2.4.2.14), respectively, was formally competitive in type; and, Horowitz and Meister [14], using CONV, also published Lineweaver–Burk plots which illustrate competitive inhibition. Therefore, despite the ultimate irreversibility of the inhibition

exerted by these agents, the constancy of these apparent kinetic patterns does support the conclusion that inhibition is occurring in two steps, the earlier of which can be wholly overcome by an infinitely high concentration of L-glutamine.

Horowitz and Meister [14] and Hartman [32] suggested that the specific mechanism by which CONV inhibits L-glutamine-utilizing enzymes is by irreversible alkylation of L-cysteinyl residues. A similar mechanism has been proposed for DON [33–35]. The suggestion that the ketoamino acids bind at L-cysteinyl residues is supported by our demonstration that dithiothreitol was capable of preventing and partially reversing the inhibition of the tumoral L-asparagine synthetase by DON, CONV and DONV.

It appears from the present studies that DON, CONV and DONV behave predominantly as L-glutamine analogs in their inhibition of L-asparagine synthetase. To see whether these agents were universal analogs of L-glutamine, their effect on other enzymes involving L-glutamine either as a substrate or as a product was investigated. DONV and CONV produced only marginal inhibition *in vitro* of L-glutamine synthetase from mouse brain and liver, while DON was totally inert. With *E. coli* L-glutamine synthetase, Wu and Yuan [36] likewise were unable to demonstrate inhibition by DON. Tiemeier and Milman [37] did, however, demonstrate that DON and DONV inhibited the synthesis of L-glutamine catalyzed by the enzyme from Chinese hamster liver. Although L-glutamine has been known to act as a product inhibitor of L-glutamine synthetase in *Bacillus subtilis* [21], *E. coli* [38] and rat liver [39], it can be conjectured that the failure of these ketoamino acids to act as analogs of L-glutamine with respect to mouse liver L-glutamine synthetase may reflect specific resistance of the enzyme from this species to product inhibition.

The fetal rat liver amidotransferases (Table 4) are closely related to mouse L-asparagine synthetase, in that they, too, utilize the carboxamide of L-glutamine. Accordingly, it was not surprising that the relative inhibitory potencies of the ketoamino acids against these enzymes mirrored their activity against L-asparagine synthetase of L5178Y/AR. DON and CONV inhibited all of the fetal rat liver amidotransferases studied, while DONV exhibited only modest inhibition of fetal rat liver L-asparagine synthetase and feeble inhibition of L-glutamine-D-fructose-6-phosphate amidotransferase. Similar inhibition of several amidotransferases by one or more of the ketoamino acids has been demonstrated by others [6, 13, 30, 33, 40].

Inasmuch as these ketoamino acids bear a structural resemblance to L-asparagine as well as to L-glutamine, it is reasonable to suppose that they might behave as L-asparagine antagonists under certain conditions. In fact, Livingston *et al.* [1] found that both CONV and DONV were strong inhibitors of L-asparaginase from mouse liver, agouti plasma and *E. coli*. DON however, was without effect on any of these enzymes. Handschumacher *et al.* [9] also showed that DONV was a potent inhibitor of *E. coli* L-asparaginase. These findings are supported by the results of the present study (Table 5); DONV inhibited all four L-asparaginase tested and CONV inhibited the hydrolases from the plasma of the squirrel monkey and from *E. carotovora*. Of great interest is the inhibition of L-asparaginase from *Erwinia* by DON. This enzyme is known to exhibit a V_{max} with L-glutamine rougly three times that of its counterpart from *E. coli*, a feature which probably serves to explain its inhibition by DON. Holcenberg *et al.* [41] also reported that DON was a good inhibitor of the mixed L-glutaminase L-asparaginase purified from the *Actinobacter* species, and a still better inhibitor of the analogous enzyme from *Pseudomonas aeruginosa*. Their data also indicate that the pseudomonal enzyme attacks DON at a rate 5000 times less than it attacks L-glutamine. In the present studies, DON has been shown to be decomposed by L-asparaginase from *Erwinia* at a similarly feeble rate.

What emerges clearly from these studies is that DONV is a potent L-asparagine antagonist and a weak analog of L-glutamine. CONV, under different circumstances, may act as either an L-asparagine or L-glutamine antagonist, while DON is the strongest L-glutamine antagonist of the three, although exhibiting L-asparagine antagonism in select systems.

Further studies on the possible therapeutic efficacy of these three agents in several experimental tumors as well as on their activity against L-asparagine synthetase *in vivo* are the subjects of companion papers [42, 43].

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