Escherichia coli L-Asparaginase. Catalytic Activity and Subunit Nature*

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ABSTRACT: The asparagine analog, 5-diazo-4-oxo-L-norvaline (DONV), has been shown to act both as a substrate for the enzyme L-asparaginase and also to inactivate the catalytic site by an irreversible reaction. The products of the catalytic reaction are 5-hydroxy-4-oxo-L-norvaline and nitrogen. Both reactions are competitively inhibited by L-asparagine, and demonstrate saturation kinetics with respect to the concentration of the analog. In the presence of 50% aqueous dimethyl sulfoxide the ability of the enzyme to decompose DONV catalytically may be fully inhibited without impairing the irreversible inactivation of the active site. The stoichiometry of binding of [5-^{14}C]DONV to asparaginase indicates that there are four active sites per molecule, a result consistent with the subunit molecular weight of 36,000 derived from acrylamide gel electrophoresis. β-Cyano-L-alanine also acts as a substrate for asparaginase. The kinetics of this nitrilase activity suggest that β-cyanoalanine interacts with the same enzyme site as do asparagine and DONV.

The L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1.) of *Escherichia coli* B, in common with L-asparaginases from other sources, is of considerable interest as an antitumor agent. These biological and clinical studies are the subject of a recent review (Cooney and Handschumacher, 1970). Whelan and Wriston (1969) have purified the enzyme and described its amino acid composition and other properties. The molecular weight as determined in their study was 133,000. Campbell and coworkers (1967) were able to distinguish two enzymes which they designated L-asparaginases 1 and 2. These enzymes differed in chemical, physical, and biological properties. Since only L-asparaginase 2 possessed antitumor activity, subsequent studies have been concentrated on this enzyme.

In an earlier report, the asparagine analog 5-diazo-4-oxo-L-norvaline (DONV)[1] has been shown to inhibit the asparaginase activity of guinea pig serum (Handschumacher *et al.*, 1968). This observation has now been extended with purified L-asparaginase prepared from *E. coli*. From this study the irreversible nature of the reaction became apparent, a result which suggested the utility of this analog for studies of the active site of this therapeutically important enzyme. However, the enzyme had the unexpected property of catalyzing the decomposition of DONV to 5-hydroxy-4-oxo-L-norvaline with the release of nitrogen gas. This decomposition reaction was much faster than the covalent binding reaction of DONV, and it was therefore not possible to inactivate and label the active site of large quantities of enzyme. This difficulty has been circumvented by selectively inhibiting the decomposition reaction with solvents. A further catalytic activity of L-asparaginase, the hydrolysis of β-cyano-L-alanine to aspartic acid and ammonia, is also discussed.

Materials and Methods

Two different L-asparaginase 2 preparations were used in this study. One of these preparations, kindly supplied by Dr. B. Berk, of E. R. Squibb and Sons, was in the form of a lyophilized powder (batch no. As335-712/15-S.7, of specific activity 180 IU/mg of protein, and batch no. OR4402-129-2L, of specific activity 220 IU/mg of protein). The other preparation, which was pure and crystalline with a specific activity of 300 IU/mg (Ho *et al.*, 1969), was the generous gift of Dr. P. Ho, of Eli Lilly and Co. (batch no. 691-H010-34). DONV and [5-^{14}C]DONV were prepared in this department by Dr. P. K. Chang, according to the method of Handschumacher *et al.* (1968). Prior to use, the radioactive DONV was separated from the polymeric material which accumulated during storage by passage through a column of Sephadex G-25, using 0.05 M potassium phosphate (pH 7.0) as eluent. Contamination of the purified [^{14}C]DONV with 5-hydroxy-4-oxo-L-norvaline, as determined by paper chromatography at 4° in ethanol–water (10:1), never exceeded 5%. β-Cyano-L-alanine, L-asparagine, and L-glutamine were obtained from Calbiochem.

To measure asparaginase activity, the production of ammonia from L-asparagine was followed by the Nessler method. The sample containing about 0.4 IU of enzyme in 0.1 ml was added to 1.9 ml of triethanolamine-HCl buffer (pH 7.6, 0.05 M) containing 2 mg of bovine plasma albumin (fraction V) and 40 μmoles of asparagine. The mixture was incubated at 37° for 30 min and the reaction was stopped by addition of 15% trichloroacetic acid (0.5 ml). After centrifugation, 0.5 ml of the supernatant fraction was added to tubes containing 7 ml of water. Nessler's reagent (1 ml) was added with rapid mixing and absorbance at 450 nm measured after 10 min. Enzymic hydrolysis of β-cyanoalanine and glutamine was assayed in the same system by substituting β-cyanoalanine or glutamine for asparagine. To determine the enzymic decomposition of

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[1] The abbreviations used in this paper are: DONV, 5-diazo-4-oxo-L-norvaline; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; IU, international unit of enzyme activity = 1 μmole of asparagine hydrolyzed per min at 37°.

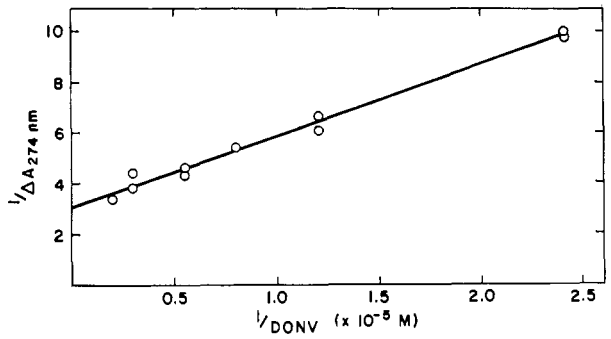

FIGURE 1: Enzymatic decomposition of DONV by asparaginase. DONV breakdown was measured by loss of absorbance at 274 nm.

DONV, 0.1 ml of a 3×10^{-3} M solution was added to 2.8 ml of 0.05 M triethanolamine-HCl buffer (pH 7.6) in a 1-cm cuvet. The mixture was equilibrated to 37° and the reaction was started by the addition of 0.05–2 IU of asparaginase in 0.1 ml of buffer. Loss of extinction at 274 nm was recorded on a Gilford Model 2000 spectrophotometer, and activity was calculated using a molar extinction coefficient of 11.0×10^3 for DONV. Because of the speed and convenience of this method it was also used as a routine assay for asparaginase activity. Radioactivity was measured in a Packard liquid scintillation counter in 20 ml of an ethanol–toluene–1,4-bis[2-(5-phenyloxazolyl)]benzene phosphor. The acrylamide gel electrophoresis was kindly performed by Dr. W. A. Summers according to the method of Weber and Osborn (1969) and the gels were scanned in a Gilford linear transport attachment.

Results

The catalytic activity of L-asparaginase from *E. coli* has previously been primarily identified with hydrolysis of the amide bonds of glutamine and asparagine. Related structures such as the hydroxamate and hydrazide are also hydrolyzed but at much slower rates, as has been shown for the *Pseudomonas* enzyme by de Groot and Lichtenstein (1960). The current work with the asparagine analog 5-diazo-4-oxo-L-norvaline reveals a new activity, the decomposition of a diazo ketone to a hydroxymethyl ketone.

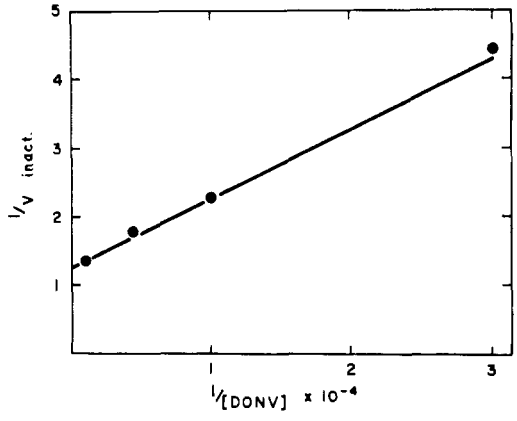

FIGURE 2: Dependence of the rate of irreversible inactivation of asparaginase upon the concentration of DONV. Experimental details are described in the text.

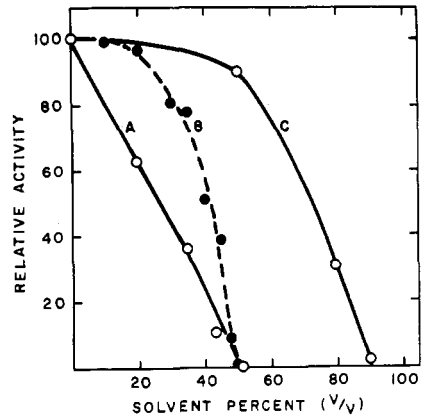

FIGURE 3: Effects of organic solvents on enzymatic decomposition of DONV. Solvent: (A) dimethylformamide, (B) Me_2SO, and (C) glycerol. Activity was measured spectrophotometrically in potassium phosphate buffer (0.05 M, pH 7.0) containing the indicated amount of solvent. The enzyme was preincubated in this medium for 10 min at 20° before assay.

Incubation of DONV with L-asparaginase results in a rapid disappearance of the characteristic diazo ketone absorbance at 274 nm with the evolution of N_2. Incubation of 1 mg of enzyme (290 IU, 0.007 μmole) in 3 ml of Tris buffer (0.1 M, pH 8.0) can decompose a total of 11 mg (70 μmoles) of DONV added in 20-μmole portions every 4 min. Ultimately the catalytic activity is eliminated by the covalent inactivation of the active site by DONV as detailed below. Heat-inactivated enzyme does not cause this decomposition. The product of the catalytic reaction has been characterized as 5-hydroxy-4-oxo-L-norvaline by paper chromatography in ethanol–H_2O (10:1) and by comparison with an authentic sample. Further identification was provided by treatment of the product (7.6 μmoles) with sodium metaperiodate (100 μmoles) which released 7.4 μmoles of formaldehyde (97%) measured by reaction with chromotropic acid (MacFadyen, 1945). The other product of this reaction, L-aspartic acid (7.4 μmoles), was identified by assay in a coupled enzyme system specific for L-aspartic acid (Cooney *et al.*, 1970).

The V_{max} of DONV decomposition (Figure 1) is only 1.9% of that for L-asparagine but the K_m is 9.5×10^{-6} M, an apparent affinity similar to that reported for asparagine, 1.2×10^{-5} M (Broome, 1968), and confirmed in this laboratory.

In the presence of high concentrations of DONV ($0.1–1.0 \times 10^{-3}$ M) and small amounts of enzyme (0.2 IU) the amount of catalytic decomposition of DONV is minimal (less than 5%) and the inactivation of the enzyme can be followed as a rate process. Under these conditions, inactivation follows saturation kinetics with respect to DONV (Figure 2) and a "K_m" of inactivation (7.3×10^{-5} M) can be obtained if inactivation velocities are calculated in the early stages of the reaction. The inactivation reaction can be inhibited in a competitive manner by L-asparagine ($1–3 \times 10^{-3}$ M) but kinetic constants for asparagine inhibition are uncertain because hydrolysis of this preferred substrate prevents exact calculation of its concentration. The apparently irreversible nature of the inactivation process was evident by the

TABLE I: Covalent Binding of DONV to Asparaginase in Presence of Organic Solvents.[a]

Inactivation Medium	DONV Concn (mM)	Final Act. (% of Initial Act.)
Phosphate buffer	0	98
55% dimethylformamide	0	57
55% dimethylformamide	2	28
90% glycerol	0	91
90% glycerol	2	10
50% Me_2SO	0	95
50% Me_2SO	2	0
50% Me_2SO	2 (+asparagine, 20 mM)	98

[a] Enzyme (20 IU) was preincubated for 10 min in 1.0 ml of phosphate buffer (0.05 M, pH 7.0), containing the indicated amount of organic solvent. DONV was added and the mixture was incubated at 20° for 30 min, then dialyzed for 12 hr against three 500-volume portions of buffer at 4°. Final activity was determined by measuring the rate of DONV decomposition.

lack of reactivation by dialysis or Sephadex chromatography in the presence of L-asparagine (10^{-3} M). The specificity of this analog for the active site of asparaginase is further illustrated by the fact that the D isomer of DONV was inactive both as substrate and inactivator of the enzyme.

To permit the use of the inactivation reaction as a technique for labeling the active site, methods were devised to dissociate the catalytic reaction from the inactivation reaction. The activity of asparaginase with either asparagine or DONV as substrate was rapidly and completely lost in concentrations of urea in excess of 5 M as previously reported for asparagine (Whelan and Wriston, 1969). Activity could be restored (96%) by dialysis for 12 hr at 4° against 1000 volumes of phosphate buffer (pH 7.0, 0.05 M). When asparaginase (24 IU in 2.0 ml of phosphate buffer, 0.05 M, pH 7) was incubated for 30 min at 20° with DONV (1 mM) in the presence of 5 M urea, and subsequently dialyzed, 97% of the initial activity was recovered. Thus, DONV was unable to bind covalently to the enzyme in the presence of 5 M urea.

The effects of varying concentrations of three organic solvents on the ability of asparaginase to decompose DONV are shown in Figure 3. Although the catalytic activity of the enzyme was completely lost in 55% dimethylformamide the enzyme was still subject to irreversible inactivation by DONV under these conditions. However, the dimethylformamide alone appeared to cause considerable permanent loss of enzyme activity in the appropriate control (Table I). Glycerol (90%) caused relatively little nonspecific inactivation of the enzyme, and binding of DONV (as measured by irreversible inactivation) proceeded more rapidly than in 55% dimethylformamide. The effect of 50% Me_2SO was of particular interest. Preincubation of the enzyme in 50% Me_2SO for 10 min at 20° caused complete loss of the catalytic activity;

TABLE II: Effect of Buffer Composition on Asparaginase Activity in 50% Me_2SO.[a]

Buffer	Act. (%)
K^+-phosphate, 0.1 M, pH 6.7	1
K^+-phosphate, 0.1 M, pH 7.8	0
Triethanolamine-HCl, 0.05 M, pH 7.6	43
Triethanolamine-HCl, 0.05 M, pH 7.6–0.1 M KCl	36
K^+-borate, 0.05 M, pH 8.0	0
Tris-HCl, 0.1 M, pH 7.5	45
Tris-H_2SO_4, 0.1 M, pH 7.5	30
Tris-H_3PO_4, 0.1 M, pH 7.5	24
HEPES–NaOH, 0.05 M, pH 7.5	33

[a] Activity is expressed as percentage of the control activity which was measured in the same buffer in the absence of Me_2SO. Asparaginase (0.4 IU) was preincubated in buffer and Me_2SO for 5 min at 20°. Activity was then assayed by measuring DONV decomposition.

subsequent removal of the Me_2SO by dialysis resulted in restoration of about 95% of the original activity. When DONV (2 mM) was added to the enzyme in the 50% Me_2SO buffer mixture, complete and rapid inactivation of the asparaginase occurred and in this case activity could not be restored by dialysis. Excess L-asparagine prevented this inactivation. Thus, although the enzyme was unable to decompose DONV under these conditions, the DONV was still able to bind to the active site.

The loss of hydrolytic activity in 50% Me_2SO was dependent upon the buffer (Table II) and for all subsequent experiments involving Me_2SO potassium phosphate (pH 7, 0.1 M) was used.

The time course of inactivation of asparaginase by DONV (100- to 200-fold molar excess) in 50% Me_2SO was pseudo first order early in the reaction; subsequently the rate of inactivation decreased. During the irreversible inactivation of the enzyme by DONV, the asparaginase, glutaminase, and DONV decomposition activities were lost at similar rates (Table III). From the initial rates of asparaginase

TABLE III: Loss of Catalytic Activities Following Treatment of L-Asparaginase with DONV.[a]

Substrate	Act. (% Initial) 1 min	5 min
L-Asparagine	67	4
L-Glutamine	69	5
DONV	63	6

[a] Enzyme (20 IU) was incubated with DONV (2 mM) at 20° for the stated time (1 or 5 min) in phosphate buffer (0.05 M, pH 7.0) containing 50% Me_2SO. The reaction mixture was diluted with 4 volumes of buffer and the activity determined after dialysis against 1000 volumes of phosphate buffer.

TABLE IV: Stoichiometry of DONV Binding to L-Asparaginase.[a]

Expt	Enzyme Prepn	Sp Act. of DONV (cpm/nmole)	Enzyme Act. (IU) Initial	Enzyme Act. (IU) Final	Enzyme Inactivated (IU)	Inactivation (%)	Radioactivity Bound (cpm)	Mole Ratio DONV:Enzyme
1	2L	324	16.8	0.7	16.1	96	537	4.1
2	691-H010-34	236	59.6	0.6	59.0	99	1404	4.0
3	S-7	236	29.5	1.2	28.3	96	688	4.1
4	S-7	195	11.9	0.1	11.8	99	268	4.6
5	691-H010-34	545	46.9	1.9	45.0	96	2690	4.4
6[b]	691-H010-34	545	46.9	16.1	30.8	66	1755	4.2
7[c]	691-H010-34	545	46.9	33.4	13.5	29	738	4.0
							Mean ± SE	4.2 ± 0.1

[a] Experimental details as described in text. The binding of DONV indicated above is corrected for nonactive site binding, as discussed in the text. Calculations are based on a molecular weight of 133,000 daltons, and an activity for pure enzyme of 300 IU/mg or 1 nmole/39.9 IU. [b] Incubation: 5 min at 0°. [c] Incubation: 1 min at 0°.

inactivation with variable concentrations of DONV a K_m of 9.5×10^{-5} M was obtained. This compared with a value of 7.3×10^{-5} M obtained in a completely aqueous system, and suggested that the presence of the 50% Me_2SO did not greatly alter the kinetics of binding to the active site.

The inactivation of asparaginase by DONV in 50% Me_2SO showed an optimum at about pH 7 (based on the pH of the buffer before addition of Me_2SO; Figure 4). When the buffer was prepared with 98% D_2O, the rate of inactivation was only 43% of that in H_2O.

These studies have permitted an investigation of the covalent binding of [5-^{14}C]DONV to the enzyme (Table IV). Asparaginase (0.04–0.20 mg) was incubated with [^{14}C]DONV (2 mM; 3 mCi/mmole) in 1 ml of 50% Me_2SO in 0.05 M potassium phosphate buffer (pH 7.0) at 20°. After 30 min a sample was assayed for enzyme activity by diluting 300-fold to eliminate the effect of Me_2SO, and the remainder dialyzed for 12 hr at 4° against three 500-volume portions of water, to remove Me_2SO and unbound [^{14}C]DONV. Protein was precipitated with an equal volume of cold 15% trichloroacetic acid and recovered after 30 min at 0° on a Millipore filter. The filter was washed with a large excess of cold 15% trichloroacetic acid and assayed for radioactivity. In each experiment a blank incubation was performed with [^{14}C]DONV but no enzyme, to correct for the small amount of trichloroacetic acid insoluble radioactive material which resulted from polymerization of DONV during the experiment. If the complete incubation was performed in the presence of asparagine (20 mM), the enzyme was completely protected from irreversible inactivation; nevertheless, under these conditions a small amount of ^{14}C was fixed to the protein (approximately 0.4 μmole/133,000 daltons) presumably by binding of DONV to the enzyme at sites other than the active center. In the experiments summarized in Table IV this small nonspecific incorporation has been subtracted from the total incorporation to give the amount of active-site labeling. The amount of analog bound was directly proportional to the amount of enzyme used, over a 5-fold range. Furthermore, binding of DONV was directly proportional to the degree of inactivation (expt 6 and 7). From these data it is concluded that four molecules of DONV are bound to each molecule of L-asparaginase (133,000 daltons).

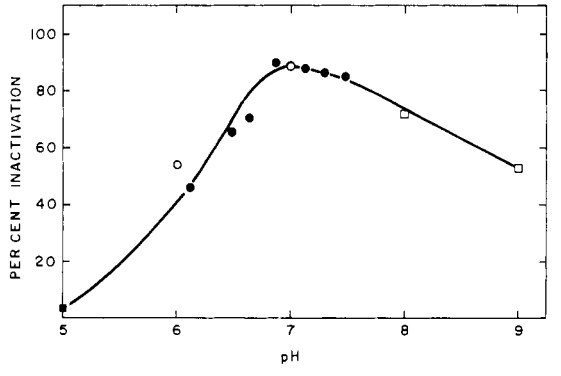

FIGURE 4: Inactivation of asparaginase by DONV as a function of pH. Enzyme (30 IU) was incubated for 2 min with DONV (2 mM) in the appropriate buffer, containing 50% Me_2SO. The reaction was stopped by a rapid 4-fold dilution with distilled water, and activity was determined after dialysis. Buffers: (■) sodium acetate, 0.05 M; (○) potassium phosphate, 0.05 M; (●) Tris-maleate–NaOH, 0.2 M; (□) potassium borate, 0.05 M.

When acrylamide gel electrophoresis of L-asparaginase was performed in the presence of 0.1% sodium dodecyl sulfate (Weber and Osborn, 1969), a single protein band was observed with a migration velocity appropriate to a molecular weight of 36,000 daltons (Figure 5). This result is consistent with a tetrameric structure and the presence of four catalytic sites as determined above.

The discovery of the catalytic ability of L-asparaginase to decompose a diazoketone prompted further studies of the substrate specificity. L-β-Cyanoalanine was found to be a poor substrate with a K_m of 5.4×10^{-3} M, and a maximum velocity 3.4% of that with asparagine as substrate. β-Cyanoalanine inhibited decomposition of DONV by asparaginase in a competitive manner, suggesting that DONV and β-cyanoalanine were hydrolyzed at the same active site (Figures 6 and 7). The apparent K_i was 4.8 ×

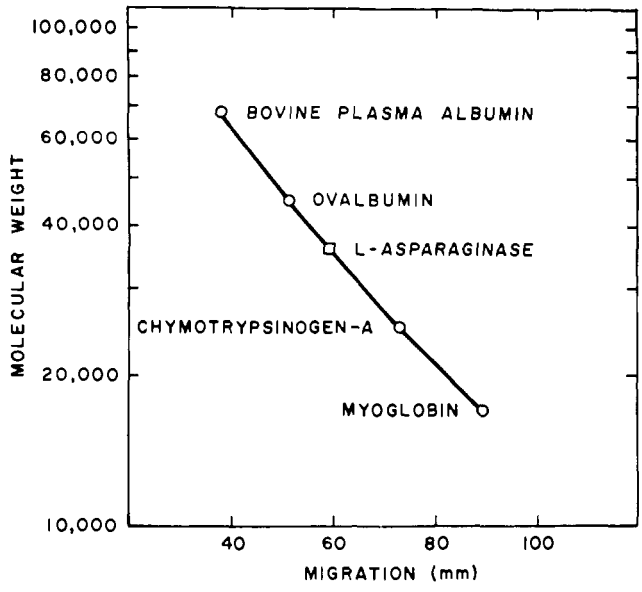

FIGURE 5: Polyacrylamide gel electrophoresis of asparaginase and molecular weight markers in the presence of sodium dodecyl sulfate. The points represent the positions of peaks as determined by densitometry.

10^{-3} M, a value in close agreement with the K_m reported above.

Others have indicated that the L-asparaginase from *E. coli* can hydrolyze glutamine (Campbell *et al.*, 1967). In this study the kinetics of this process were determined for comparison with the above substrates. A K_m of 2.7×10^{-3} M was observed, and with saturating levels of glutamine the maximal velocity was 2.6% of that with asparagine as substrate.

Discussion

DONV appears to react with asparaginase in three distinct ways. First, it can bind covalently at or near the catalytic center, causing permanent inactivation of the enzyme. As the data in Table IV demonstrate, loss of activity is directly proportional to the amount of DONV bound at the active sites. A small amount of DONV is bound to the enzyme, however, even in the presence of high concentrations of asparagine which completely protect the enzyme from inactivation; this incorporation (about 9% of the amount of DONV bound in the absence of asparagine) presumably represents binding of DONV to the enzyme at sites other than the catalytic center.[2] Finally, DONV is decomposed by asparaginase. This reaction is over 400 times faster than the irreversible binding reaction. Thus, to inactivate relatively large amounts of enzyme, it was necessary to inhibit specifically the catalytic reaction by the use of 50% Me_2SO. The

[2] That such nonspecific binding of DONV to protein may occur was also shown by an experiment in which crystalline bovine plasma albumin was incubated with [^{14}C]DONV under experimental conditions exactly the same as for the asparaginase-labeling experiment. A small incorporation of isotope was found, equal to about 0.4 mole of DONV/mole of protein.

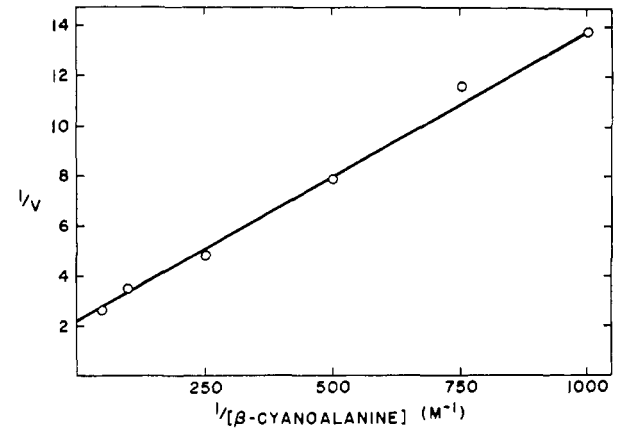

FIGURE 6: Hydrolysis of β-cyano-L-alanine by asparaginase. Activity was determined by the Nessler method as described in the text.

specific mechanism of this Me_2SO effect is not known; possibly it is a consequence of the weakening of hydrophobic bonds. The K_m for the binding of DONV in 50% Me_2SO was not greatly different from that in aqueous medium, suggesting that the conformation of the active site was not drastically altered; however, the V_{max} was greatly increased. Treatment with 5 M urea caused sufficient disruption of the conformation that DONV could no longer combine with the active site.

Although DONV binding and DONV decomposition presumably proceed *via* a common enzyme–substrate complex, the K_m for the covalent binding reaction was almost an order of magnitude higher than for the hydrolytic reaction. This suggests that a second dissociable complex may be involved in the inactivation reaction. That the enzyme–DONV complex has two alternative fates suggests that decomposition and covalent binding either involve attack by different reactive groups in the active site or that a single group can interact with the DONV in two distinct ways. Since both covalent binding and hydrolysis of DONV proceed considerably

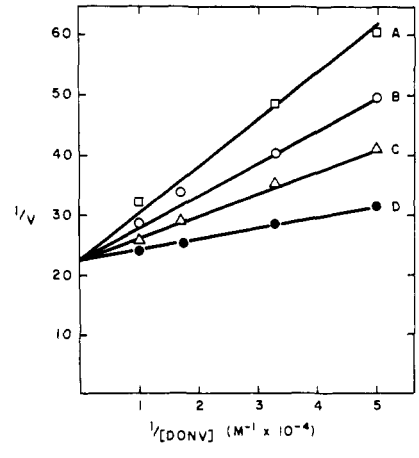

FIGURE 7: Inhibition of the enzymatic decomposition of DONV as a function of β-cyanoalanine concentration. Activity was measured spectrophotometrically at 274 nm.

more slowly in 98% D_2O, both reactions may involve a rate-limiting proton transfer.

From these binding studies, it can be concluded that there are four active sites in each molecule of L-asparaginase of mol wt 133,000. The molecular weight of the enzyme as determined on sodium dodecyl sulfate–acrylamide gel electrophoresis is consistent with the proposed tetrameric structure. Other evidence suggestive of a subunit structure has been reported by Kirchbaum *et al.* (1969), Frank and Veros (1969), and Greenquist and Wriston (1970). Highly purified preparations analyzed by Frank and Ho appeared to dissociate into subunits with a molecular weight between 30,000 and 40,000, a result consistent with the existence of 4 subunits (B. H. Frank and P. P. K. Ho, personal communication). Analysis of disulfide groups indicates 4 such units/133,000 daltons (R. C. Jackson and R. E. Handschumacher, 1970, unpublished data). The complex kinetics of DONV binding found in the present study suggest that when some of the active sites have been inactivated, the affinity of the remaining sites for DONV is reduced. This negative cooperativity is consistent with an enzyme composed of subunits. The specific binding of [^{14}C]DONV in the region of the active site provides a means for the investigation of the amino acid sequence near the catalytic center. Such a study is now in progress.

The multiple substrate specificity of this therapeutically effective enzyme, as previously reported (Campbell *et al.*, 1967; de Groot and Lichtenstein, 1960) and extended in this report to include β-cyanoalanine as well as 5-diazo-4-oxo-L-norvaline, suggests the possibility of multiple mechanisms being operative in the same general substrate site.

Acknowledgments

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