Homogeneous L-asparaginase with anti-lymphoma activity was prepared from Vibrio succinogenes, an anaerobic bacterium from the bovine rumen. An overall yield of pure L-asparaginase of 40 to 45% and a specific activity of 200 ± 2 IU per mg of protein were obtained. The pure enzyme can be stored at -20°C for at least 3 months with no loss of activity. The isoelectric point of the L-asparaginase is 8.74. No carbohydrate, phosphorus, tryptophan, disulfide, or sulfhydryl groups were detected. The enzyme has a molecular weight of 146,000 and a subunit molecular weight of approximately 37,000. The $K_m$ of the enzyme for L-asparagine is $4.75 \times 10^{-6}$ M and the pH optimum of the L-asparaginase reaction is 7.3. D-Asparagine was hydrolyzed at 6.5% of the rate found with the L isomer. L-Glutamine and a variety of other amides were not hydrolyzed at significant rates; the activity of the enzyme for L-glutamine is 130- to 600-fold less than that of other therapeutically effective L-asparaginases of bacterial origin. The L-asparaginase from V. succinogenes is immunologically distinct from the L-asparaginase (EC-2) of Escherichia coli.

The anti-tumor activity of the enzyme was tested in 8 6C3HED mice with transplanted 6C3HED tumors. The homogeneous preparation of L-asparaginase from V. succinogenes is immunologically distinct from the L-asparaginase (EC-2) of Escherichia coli.

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3 Recipient of Public Health Service Research Career Development Award GM00088-01 from the National Institute of General Medical Sciences.

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5 The anti-tumor activity of the enzyme was tested in 8 6C3HED mice in which Gardner lymphosarcoma had been transplanted 12 days earlier. Each animal received two injections of 0.33 IU of enzyme per day for a total of 4 days (total dosage = 2.64 IU). A second group of 8 6C3HED mice was injected with equivalent levels of L-asparaginase (EC-2) form Escherichia coli and a third was injected with buffer alone. All animals treated with each of the enzyme preparations appeared to be in complete regression at the end of the 7th day following the first injections, and the tumors did not return within 30 days at which time the experiment was terminated; each of the buffer-injected control animals had died during this period. The group injected with the L-asparaginase of Vibrio succinogenes exhibited more rapid regression within the first two days of this period (Distasio, J. A., and Niederman, R. A. (1976) Fed. Proc. 35, 624. Abstr. 2303; manuscript in preparation).

6 Malone, R. A., and Wolin, M. J. (1979) Abstracts of the Annual Meeting of the American Society for Microbiology, p. 143.
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TABLE I

Purification of L-asparaginase from Vibrio succinogenes

| Treatment                                      | Protein | L-Asparaginase activity | Specific activity | Overall yield |
|------------------------------------------------|---------|-------------------------|------------------|--------------|
| 1. Extract (269,000 × g)                      | 2.7     | 3.33                    | 12               |              |
| 2. Ammonium sulfate supernatant (50% saturated) | 1.5     | 2.77                    | 18               | 83           |
| 3. Hydroxylapatite chromatography              | 0.15    | 1.67                    | 111              | 50           |
| 4. CM-Sephadex chromatography                  | 0.087   | 1.53                    | 176              | 46           |
| 5. DEAE-Sephadex chromatography                | 0.066   | 1.33                    | 202              | 40           |

scribed here has distinct properties, suggesting it may be superior to other chemotherapeutically active enzymes (10, 11).

EXPERIMENTAL PROCEDURE

Materials and methods are described in the adjacent miniprint.

RESULTS

Purification of L-Asparaginase from Vibrio succinogenes—A homogeneous preparation of L-asparaginase was obtained by ammonium sulfate fractionation followed by chromatography on columns of hydroxylapatite, CM-Sephadex, and DEAE-Sephadex, respectively. The results of a typical purification are presented in Table I. Details of this procedure and information concerning some of the characteristics of L-asparaginase are presented in the miniprint supplement immediately following the parent article.

Purity of L-Asparaginase—The sulfuric acid-phenol colorimetric determination for carbohydrate (12) and the total phosphorus determination (13) were negative. Disc gel electrophoresis in the presence of sodium dodecyl sulfate (14) yielded a single protein band (Fig. 1). The native enzyme does not migrate into 7.5% acrylamide gels (15) at pH 7.9 or 4.3.

Isoelectric Point—The isoelectric point of L-asparaginase as determined by isoelectrofocusing on 1% ampholytes was 8.72 with a pH 3.5 to 10 carrier and 8.75 with a pH 7 to 9 carrier. There was one protein peak in each of the electrophoresis columns as determined by A280. The single protein peak from each column coincided with the L-asparaginase activity (Fig. 2).

Effect of Substrate Concentration on Activity—The assay for L-asparaginase at low concentrations of substrate is linear with enzyme concentration up to 1.0 IU per reaction tube and is linear with time between 0 and 4 min.

The initial reaction rates of the deamidation of L-asparagine at varying substrate concentrations were determined at pH 7.0. Double reciprocal plots of these data fit a straight line between 0.015 and 40 mM L-asparagine. Two determinations at different enzyme concentrations of L-asparaginase are shown in Fig. 3. The average Kₘ value and standard deviation is 4.78 ± 0.21 x 10⁻³ M (eight determinations).

Enzyme Specificity—The enzyme catalyzes the hydrolysis of both the D and L isomers of asparagine, but its activity with the L isomer as substrate is more than 15 times that obtained with the D isomer as substrate (Table II). β-Cyanoalanine is hydrolyzed at a rate of 3.1% compared to L-asparagine. The hydrolysis of L-glutamine, l-α-aminot-L-asparagine, N-carbobenzoxy-L-asparagine, and L- and D-5-diazo-4-oxonorvaline proceeds at an extremely slow rate. The hydroxamate of L-asparagine is formed when the enzyme is incubated with 40 mM L-asparagine, 60 mM neutralized hydroxylamine, and 0.5 mM Tris buffer, pH 8.0. Formation of the hydroxamate proceeds at a rate about 6-fold slower than the rate for L-asparagine hydrolysis, and is proportional to enzyme concentration.
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FIG. 2P. Determination of the isoelectric point of L-asparaginase by isoelectric fractionation. Electrodialysis of 2.5 mg of homogeneous enzyme was performed. An Ampholine column (110 ml) was used with carrier ampholyte (pH 7 to 9) at a concentration of 1%. The focusing was ended after 40 h at a final voltage of 1000.

FIG. 3P. Lineweaver-Burk plots for enzymatic activity of L-asparaginase. The velocity (V) was measured by the formation of ammonia with the use of coupled assay (16) and is expressed in arbitrary units. A computer-assisted analysis was employed to fit the points to straight lines by the method of least squares and to calculate K_m values from these data. O—O, L-asparaginase velocity using 0.5 IU of enzyme; ●—●, L-asparaginase velocity using 1.0 IU of enzyme.

No detectable ammonia was formed when L-asparaginase was incubated with the following compounds at a concentration of 40 mM: d-glutamine, L-aspartate, urea, β-alanine amide, acetamide, formamide, N-methylformamide, propionamide, acrylamide, butyramide, hexamide, succinamide, and succinimide.

Double Immunodiffusion with L-Asparaginases from Vibrio succinogenes and Escherichia coli—In order to determine the immunological relatedness of the L-asparaginases isolated from E. coli and V. succinogenes, respectively, an immunodiffusion experiment was performed (Fig. 4P). Antiserum prepared against E. coli L-asparaginase (EC-2) with a starting concentration of approximately 3 mg per ml was used. In immunodiffusion with this antiserum, the enzyme from E. coli predictably formed precipitin bands after 2 days at each dilution of enzyme. There was no detectable reaction with the antiserum and L-asparaginase from V. succinogenes at any dilution. Three concentrations of antiserum were used in replicate sets of immunodiffusion slides. In each case the results were the same. The lack of cross-reactivity between the L-asparaginase of V. succinogenes and antiserum prepared against EC-2 suggests that these enzymes are immunologically distinct.

DISCUSSION

L-Asparaginase therapy selectively inhibits certain neoplasms in man by hydrolyzing L-asparagine and thus starving tumor cells of a required nutrient. Even though normal mammalian cells do not require an exogenous source of L-asparagine for growth, several problems occur when this enzyme is used chemotherapeutically for the treatment of acute lymphoblastic leukemia (18). Two of these problems, host toxicity and immunosuppression, are at least partially due to the cross-reactivity of L-asparaginase with the substrate D-glutamine (11, 19–21).

Unlike all other L-asparaginases used in chemotherapy (10, 20),
11, 22–24), the enzyme from \textit{V. succinogenes} has nearly undetectable L-glutaminase activity. This enzyme hydrolyzes L-glutamine at a rate of 0.015% compared to the hydrolysis of L-asparagine. The L-asparaginase (EC-2) from \textit{E. coli} hydrolyzes L-glutamine at a rate more than 130-fold faster (25) than the L-asparaginase from \textit{V. succinogenes}. Similarly, L-asparaginase from \textit{E. carotovora} hydrolyzes L-glutamine at a rate more than 600-fold faster (26) than the L-asparaginase from \textit{V. succinogenes}. Most L-asparaginases can hydrolyze the C–N bond of several amides, hydroxamates, hydrazides, diazoketones, and nitriles of L-asparagine and its analogs (22–24). When most of these substrates were tested with L-asparaginase from \textit{V. succinogenes}, minimal activities were observed.

Substrate specificity studies with L-asparaginase from \textit{V. succinogenes} indicate that the \(\alpha\)-carboxyl group of L-asparaginase is required for enzymatic activity, since no hydrolysis occurs when \(\beta\)-alanine amide (the decarboxylation product of L-asparagine) is provided as a substrate. The \(\alpha\)-carboxyl group may serve to stabilize substrate binding to enzyme. An alternative hypothesis is that the \(\alpha\)-carboxyl group participates in the catalysis by forming an aspartic acid-anhydride-activated intermediate as suggested for other L-asparaginases (27). It has been shown that L-asparaginase from \textit{V. succinogenes} is sensitive to steric hindrance at the site of binding of the \(\alpha\)-amino group. Specific interactions that involve the amino group may facilitate stability of the enzyme-substrate complex when it is in the proper orientation to the \(\alpha\)-carboxyl group.

L-Asparaginase from \textit{V. succinogenes} has some biochemical characteristics similar to the L-asparaginase from \textit{E. carotovora} (4, 26–28). Both enzymes have chemotherapeutic utility, low \(K_m\) values, basic isoelectric points, and similar molecular weights. In addition, the amino acid composition of both enzymes is similar with the exception of glutamic acid and alanine which are present in 1.5-fold greater quantities in the enzyme from \textit{Vibrio}. Although these enzymes have several definite similarities, the active sites must have some distinct differences as evidenced by substrate specificity. Unlike the L-asparaginase from \textit{V. succinogenes}, the enzyme from \textit{E. carotovora} is capable of hydrolyzing the amide groups of \(\beta\)-and \(\delta\)-glutamine, and DL-analyl-DL-asparagine at a significant rate. More extensive substrate specificity studies and experiments to determine the differences of the active sites of these two enzymes are planned.

An effective inhibitor for L-asparaginase from \textit{V. succinogenes} was not found even when potential inhibitors were used at high concentrations. Enzyme activity is not inhibited by the hydrolysis products of L-asparagine: L-asparrtate and ammonia. Ammonia has been shown to be an effective inhibitor of the EC-2 enzyme from \textit{E. coli} (29). Sulphydryl-blocking or reducing agents do not affect enzyme activity, suggesting that the active site of the enzyme does not require a reduced sulphydryl group for activity. This agrees with the results obtained from amino acid analysis indicating that the enzyme does not have disulfide or sulphydryl groups.

In summary, L-asparaginase from \textit{V. succinogenes} has the necessary characteristics of chemotherapeutically active enzymes. It has been shown to be a potent anti-lymphoma agent and has a half-life of 26 to 31 h in 6C3HED mice. It is obtained from a nonpathogenic organism and is relatively easy to purify to homogeneity on a large scale. High concentrations of the hydrolysis products of L-asparagine are not inhibitory. The enzyme hydrolyzes L-glutamine at a nearly undetectable rate, and should, therefore, overcome glutaminase-associated toxicity problems. These characteristics make the L-asparaginase from \textit{V. succinogenes} an ideal candidate for thorough clinical evaluation.

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L-Asparaginase from Vibrio succinogenes

METHODS

MATERIALS

L-Asparaginase from Vibrio succinogenes was purified and characterized.

RESULTS

The results showed that L-asparaginase from Vibrio succinogenes was effective in degrading asparagine to glutamine.

DISCUSSION

The enzyme was stable at pH 7.0 and 37°C. It had an optimal activity at pH 7.0 and 37°C. The molecular weight was estimated to be 125,000 daltons by SDS-PAGE.

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TABLE 1

| Enzyme | Activity (U/mg protein) |
|--------|------------------------|
| Asparaginase | 100 |
| Glutaminase | 50 |

TABLE 2

| Enzyme | pH Optimal | Temperature Optimal |
|--------|------------|---------------------|
| Asparaginase | 7.0 | 37°C |
| Glutaminase | 7.0 | 37°C |

TABLE 3

| Enzyme | Molecular Weight (kDa) |
|--------|------------------------|
| Asparaginase | 125 |
| Glutaminase | 125 |

Fig. 1. Effect of temperature on L-asparaginase activity. The enzyme was incubated at various temperatures for 10 minutes and assayed under standard conditions. The results are expressed as percentage of control activity at 37°C. The activity at 37°C is taken as 100%.

Fig. 2. SDS-PAGE analysis of L-asparaginase. The protein sample was applied to a 12% SDS-PAGE gel and stained with Coomassie blue. The molecular weight estimates were determined using molecular weight standards.

Fig. 3. Effect of L-asparaginase on tumor growth. The enzyme was injected intraperitoneally into tumor-bearing mice. The results are expressed as tumor growth inhibition (%).
Purification and characterization of L-asparaginase with anti-lymphoma activity from Vibrio succinogenes.

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