Purification and Properties of L-Asparaginase from *Serratia marcescens*¹

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The purification and properties of a tumor inhibitory L-asparaginase from *Serratia marcescens* are described. The following properties of the enzyme were examined: kinetics of the enzyme reaction, catalytic activity as a function of pH, boundary sedimentation velocity, electrophoresis on polyacrylamide gel, immunoelectrophoresis against homologous and heterologous antisera, immunodiffusion, blood clearance rate in mice, and inhibition of the 6C3HED lymphoma in C3H mice. Complete regression of this tumor was obtained with a smaller dose of the enzyme from *S. marcescens* than with enzyme from *Escherichia coli*. The reason for this difference was not evident from a comparison of several properties of the two enzymes.

Kidd (16) discovered that guinea pig serum inhibited the growth of certain murine tumors, and Broome (3) identified the tumor inhibitory substance as L-asparaginase (L-asparagine amidohydrolase, EC3.5.1.1). The following microbial sources of L-asparaginase with tumor inhibitory activity have been described: *Escherichia coli* (2, 19, 30-32, 39), *Serratia marcescens* (33), *Erwinia aroideae* (26), *Erwinia carotovora* (23, 36), *Aspergillus terreus* (6), and *Mycobacterium tuberculosis* (14, 27). The L-asparaginases from *Bacillus coagulans* (19) and *Saccharomyces cerevisiae* (4) were not inhibitory to tumor growth. *E. coli* strains produce two L-asparaginases, one with and the other without tumor inhibitory activity (5, 34); the two enzymes can be separated by chromatography. The properties of crystalline *E. coli* asparaginase were studied in some detail (1, 10, 13, 35, 37). More recently, two isozymes of *E. coli* L-asparaginase with tumor inhibitory activity were crystallized (1). We describe here the partial purification of L-asparaginase from *S. marcescens* and some comparative properties of the enzyme with those of *E. coli* asparaginase. Preliminary reports of this work have appeared (J. W. Boyd and A. W. Phillips, Bacteriol. Proc., p. 105, 1968; p. 137 and p. 153, 1969).

MATERIALS AND METHODS

Bacterial strain. *S. marcescens* ATCC60 was maintained by cultivation on Trypticase Soy Agar slants at 28 C.

Growth and harvesting of bacteria. Cells were grown in a medium containing 4% (w/v) autolyzed yeast (Nestlé Co., White Plains, N. Y.) at an initial pH 5.0 as described previously (11, 12). Each 500-ml Erlenmeyer flask containing 100 ml of inoculated medium was incubated at 26 C on a rotary shaker for 40 hr. Cells were harvested by centrifugation, and approximately 25 g (wet weight) of nonpigmented cell paste, containing about 1.7 units of L-asparaginase per g, was obtained per liter of culture.

Enzyme assays. Routine L-asparaginase assays were conducted by a modified method based on that of Meister et al. (20). Portions (1 to 100 μ liters) of enzyme solution were added to 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.4) to give a final volume of 1.5 ml. The reaction was initiated by the addition of 0.5 ml of 0.04 M L-asparagine in the same buffer and conducted at 37 C in a reciprocal water bath shaker. The reaction was stopped by the addition of 0.1 ml of 1.5 M trichloroacetic acid. If necessary, the mixture was centrifuged to remove precipitated proteins. Ammonia released in the reaction was determined by the addition of Nessler's reagent to the diluted supernatant fluid and, after 15 min, observing the absorbancy at 500 nm. Our studies on enzyme kinetics utilized a reduced nicotinamide adenine dinucleotide (NADH)-dependent coupled assay for ammonia producing systems (15). NADH and ammonia are required in equimolar amounts for the synthesis of glutamate from α -ketoglutarate by glutamic dehydrogenase. The rate of ammonia production from L-asparaginase may be calculated from the rate of NADH oxidation

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as determined spectrophotometrically at 340 nm. The reaction mixture contained 0.8 μ mole of α -ketoglutarate (10 μ liters), 0.25 μ mole of NADH (100 μ liters), 0.67 mg of glutamic dehydrogenase (50 μ liters), and different amounts of L-asparaginase and L-asparagine. All reagents were made up in 0.05 M Tris-hydrochloride buffer (pH 7.4), and the total volume of the reaction mixture was brought to 1.0 ml with buffer. The reaction was initiated by the addition of L-asparaginase. The rate of the coupled reaction at 37 C was determined from the linear and maximal slope of the curve describing the rate of NADH oxidation taken from the Gilford recording spectrophotometer. The assay of the enzyme in whole blood was performed after Broome (4).

Definition of enzyme unit. In this report, the unit of L-asparaginase activity is defined as that amount of enzyme which liberates 1 μ mole of ammonia per min at 37 C under conditions of the assay [1 International Unit (IU)].

Protein assays. Analyses for protein were carried out by the method of Lowry et al. (18), by spectrophotometric assay at 260 and 280 nm by the method of Warburg and Christian (38), and by spectrophotometric assay at 215 and 225 nm (21).

Animals. Female C3H/HE mice, 6 to 8 weeks old, were obtained from the Jackson Memorial Laboratories, Bar Harbor, Me., and from the Texas Inbred Mouse Co., Houston, Tex. New Zealand white doe rabbits, weighing 2 to 3 kg, were used in the production of antisera.

Tumor cells. The Gardner lymphosarcoma 6C3HED in the C3H mouse was a gift from John G. Kidd and was maintained by transplantation. Solid subcutaneous tumors were removed under aseptic conditions 7 to 9 days after implantation, cut into pieces (1 mm²), and implanted subcutaneously by trocar into the shaved flank of young adult C3H mice. A palpable tumor occurred within 7 days, which became enlarged and eventually metastasized; death usually occurred within 30 days after tumor implantation. The rate of tumor growth or regression was determined from caliper measurements on tumors in three diameters.

Purification of *S. marcescens* L-asparaginase. *S. marcescens* cells were washed twice with 0.05 M Tris buffer (pH 8.6) and suspended in two volumes of cold buffer. All steps in purification were carried out at 4 to 8 C. The slurry was sonically oscillated at maximum power in a Bronwill Biosonic II apparatus, and the material was centrifuged at 16,300 $\times$ g for 20 min. The supernatant fluid was heated with stirring to 50 C for 5 min and then rapidly cooled. The precipitate was removed by centrifugation for 20 min at 30,000 $\times$ g. To the supernatant fluid was added 0.05 to 0.1 volume of 1.0 M MnCl₂. The mixture was stirred for 2 hr before centrifugation at 30,000 $\times$ g; the precipitate was discarded. The preparation was adjusted to pH 8.8 with 3 M KOH, and the precipitate was removed by centrifugation. The enzyme preparation was then frozen and thawed slowly three to four times, with centrifugation after each thawing to remove precipitates which were discarded.

Diethylaminoethyl (DEAE) cellulose (Whatman

microgranular DE-52, 1.0 meq/g) was prepared as recommended by the manufacturer and placed in a chromatographic column (4.2 by 25 cm) containing 300 ml of settled bed volume. A solution containing 3 to 5 g of protein was passed through the column after equilibration with 0.01 M Tris containing 0.01 M KCl. The column was washed with 5 to 10 volumes of the same buffer and eluted with 0.01 M Tris containing 0.05 M KCl. Ten-milliliter fractions were collected and assayed for protein and enzyme activity. Pooled fractions with high specific activity were further purified by ammonium sulfate fractionation. Additional purification was done on an hydroxylapatite (Bio-Gel HT, BioRad Laboratories, Richmond, Calif.) column (1 by 25 cm) containing 45 ml of settled bed volume. The column was charged with about 50 mg of protein after equilibration with 0.01 M potassium phosphate buffer (pH 6.9), washed with several column volumes of 0.05 M phosphate buffer, and finally eluted with 0.10 M potassium phosphate. Fractions containing high specific activities were pooled and further purified on polyacrylamide gel disc electrophoresis on a Poly-Analyt Apparatus (Buchler Instruments, Inc., Fort Lee, N.J.). The methods of Davis (9) were followed. Experiments were performed at pH 9.3 by using separation gels of 4.5 or 7.5% for 1 to 2 hr at 2.5 ma per tube. Gels were stained with 1% aqueous Coomassie blue, diluted 1:20 in 12.5% trichloroacetic acid for 1 hr, and stored in 10% trichloroacetic acid after Chrambach et al. (8). For preparative separations using 0.2 mg of protein per gel, a representative gel was stained for the location of bands, and the other gels were examined under ultraviolet light for location of protein bands which were then removed. The crushed gel was eluted with 0.01 M sodium phosphate buffer (pH 6.9) and dialyzed to remove contaminating gel constituents. The enzyme was usually stored frozen in the elution buffer.

Preparation of antisera against L-asparaginase. Antisera against L-asparaginase were prepared with antigens containing 67 IU of L-asparaginase per mg of protein and emulsified in Freund's complete adjuvant. Rabbits received two intramuscular doses per week for 3 weeks (50 mg of protein). Animals were bled 10 to 12 days after the last injection, and the clear antisera were stored frozen without preservative.

Immunoelectrophoresis. Immunoelectrophoresis was conducted on silicone-treated slides prepared with 3 ml each of 0.75% Ionagar no. 2 (Consolidated Laboratories, Inc., Chicago, Ill.) in an electrophoresis apparatus (National Instrument Co.) by using 0.01 M Veronal buffer (pH 8.6). A current of 50 ma per eight slides was usually employed for 2 to 2.5 hr.

Ultracentrifugal analyses. Boundary sedimentation velocity analyses were carried out in a Beckman model E analytical ultracentrifuge (Spinco Division, Palo Alto, Calif.) by using schlieren optics. Centerpieces of 12 mm were used in an AND-1869 rotor, and two different enzyme preparations were placed in separate cells with one cell equipped with a 1° positive radial wedge to cast both schlieren patterns on the plate. Enzyme preparations were dialyzed against 0.01 M sodium phosphate buffer (pH 6.9) before sedimentation analyses. A typical experiment was performed at 56,100 rev/min at

9 C, and the schlieren patterns were photographed at 8-min intervals. Sedimentation velocity was determined from measurements of the schlieren patterns on a Nikon profile projector.

Lactic dehydrogenase assay. The lactic dehydrogenase activity of mouse blood was measured by the method described by Neilands (22) and is based upon the change in absorbancy at 340 nm accompanying the reduction of nicotinamide adenine dinucleotide. The reaction was linear over a 6-min interval, and the slope was determined from the curve taken from a recording spectrophotometer. One unit of lactic dehydrogenase activity is defined as an increase in absorbancy of 0.001 per cm per min.

Lactic dehydrogenase virus. A partially purified preparation of the lactic dehydrogenase virus containing 10^7 median infective doses (ID_{50}) per ml was kindly supplied by Abner L. Notkins, National Dental Institute.

Chemicals. Chemicals were obtained from the following suppliers: L-asparagine from Calbiochem; Tris, certified primary standard, from Fisher Scientific Co.; ammonium sulfate, enzyme grade, from Mann Research; hydroxylapatite, Bio-gel HT, from Bio-Rad Laboratories; mercuric iodide from Fisher Scientific Co.; ingredients for the NADH-dependent coupled assay for asparaginase, from Sigma Chemical Co.; other chemicals, from Mallinckrodt.

RESULTS

Purification of L-asparaginase from *S. marcescens*. The purification of L-asparaginase from *S. marcescens* is summarized in Table 1. The procedure described yielded a 365-fold purification and 15% recovery of the enzyme. Enzyme preparations were found to be more stable if they were purified on a DEAE column before ammonium sulfate fractionation. A typical elution profile from a DEAE column is shown in Fig. 1. Fractions with maximal activity were pooled for ammonium sulfate fractionation; this pool contained 0.2 to 0.6 mg of protein per ml. Approximately 80 to 90% of the enzyme activity was salted out at 55 and 65% saturation of ammonium sulfate. The precipitate was dialyzed against 0.01 M sodium phosphate (pH 6.9) and

loaded on an hydroxylapatite column. The elution profile from this column is shown in Fig. 2. Fractions with high enzyme activity were pooled and concentrated in a Diaflo apparatus (Amicon Corp., Lexington, Mass.), and the enzyme preparation was purified by gel electrophoresis with recoveries of 80 to 95%. The dialyzed eluate from the gel electrophoresis was applied to a G-200 Sephadex column (0.9×15 cm) to remove nondialyzable gel constituents and was eluted with 0.01 M sodium phosphate buffer (pH 6.9). The final enzyme preparation was concentrated by dialysis against cold solid Sephadex G-200, and the solution was stored frozen or lyophilized.

Analytical gel electrophoresis of L-asparaginase. Analyses were performed on L-asparaginase by analytical disc electrophoresis. The protein bands in the various enzyme preparations are shown in Fig. 3. Gel Id shows at least two proteins in *E. coli* L-asparaginase of high specific activity (300 IU/mg of protein). Gels IIc and IId in Fig. 3 contain the eluate of the major band of gel IId. To test the possibility that these slower moving minor bands were aggregation products of what was assumed to be an essentially homogeneous preparation, a sample of the enzyme preparation was treated with 0.1% recrystallized sodium dodecyl sulfate (SDS) before electrophoresis. Gel IIe in Fig. 3 shows that the SDS treatment eliminated the slower moving bands to yield a single band upon electrophoresis. This observation, together with other data, supported the notion that our *S. marcescens* enzyme preparations with specific activity of 250 IU per mg of protein were homogeneous materials. On the other hand, SDS treatment of *E. coli* L-asparaginase with a specific activity of 300 IU/mg failed to remove the minor, slow moving protein which was always seen in this preparation (Fig. 3, gel Ie). In Fig. 3, gel IIIf shows the pattern obtained from an enzyme fraction from an hydroxylapatite column with a specific activity of 360 IU/mg. A band of contaminating

TABLE 1. Purification of *Serratia marcescens* L-asparaginase

Purification step	Total protein (g)	Total units (IU) ^a	Specific activity (IU/mg of protein)	Enrichment (fold)	Total recovery (%)
Crude extract	30.0	21,000	0.7		100
Heat; MnCl ₂	7.64	15,017	2.0	2.8	72
KOH; freeze-thaw	5.58	14,872	2.7	3.8	71
DEAE cellulose chromatography	0.113	5,025	44.5	63.5	24
(NH ₄) ₂ SO ₄ fractionation	0.048	3,467	71.7	102.0	17
Hydroxylapatite chromatography	0.016	3,133	200.0	286.0	15
Polyacrylamide gel electrophoresis	0.012	3,100	255.0	365.0	15

^a IU, International Units.

FIG. 1. DEAE-cellulose chromatography of *Serratia marcescens* L-asparaginase fraction from freeze-thaw treatment. The column was equilibrated and washed with 0.01 M Tris-hydrochloride buffer (pH 8.6) containing 0.05 M KCl. Elution was begun at arrow with the same buffer made 0.1 M in KCl. The points represent enzyme activity and the line represents protein.

FIG. 2. Hydroxylapatite chromatography of *Serratia marcescens* L-asparaginase preparation from ammonium sulfate fractionation. The column was equilibrated, loaded, and washed with 0.01 M sodium phosphate buffer (pH 6.9). The first arrow indicates a change in buffer to 0.05 M. The second arrow indicates elution with 0.10 M buffer. The pH was constant. Symbols: O, enzyme activity; ●, protein.

protein with greater mobility than the enzyme protein can be seen in Fig. 3.

Ultracentrifugal analyses of L-asparaginase. Boundary sedimentation velocity analyses of preparations of L-asparaginase from *S. marcescens* and *E. coli* were conducted at enzyme protein concentrations of 1.0 to 10.0 mg/ml. Enzyme solutions were first dialyzed against 0.01 M sodium phosphate buffer at pH 6.9. Figure 4 shows schlieren plates from representative experiments. Plots of the logarithm of the distance of the peak from the center of rotation against time (8-min intervals) yielded a straight line. The sedimentation coefficient was calculated from the slope of the plot in Fig. 4 and was corrected for

FIG. 3. Electrophoresis of L-asparaginase in polyacrylamide gel. I, *Escherichia coli* enzyme in 7.5% gel except as noted: specific activities of (a) 74, (b) 74 in 4.5% gel, (c) 124, (d) 300 in 4.5% gel, and (e) 300 after treatment with 0.1% sodium dodecyl sulfate. II, *Serratia marcescens* enzyme in 7.5% gel except as noted: specific activities of (a) 70, (b) 200, (c) 250, (d) 250 in 4.5% gel, (e) 250 after treatment with 0.1% sodium dodecyl sulfate, and (f) 361 fraction from hydroxylapatite column.

solvent viscosity and temperature to standard conditions ($S_{20, w}$), water at 20 C, using the partial specific volume of an average protein (0.749 at 20 C; 0.754 at 30 C). Figure 5 shows plots of the dependence of the sedimentation coefficient on protein concentration for the enzyme from *S. marcescens* and from *E. coli*. The sedimentation coefficient extrapolated to infinite dilution at standard conditions ($S_{20, w}$) was determined to be 7.6S ($S = 10^{-13}$ cm/sec) for both enzymes.

Effect of pH on enzyme activity. The influence of pH on enzyme activity is shown in Fig. 6. The pH activity curves for L-asparaginase from *E. coli* and *S. marcescens* are similar above pH 7.0; the curve for the *S. marcescens* enzyme remains in somewhat of a plateau between pH 6.2 and 6.8, in contrast to a definite decrease in activity of the *E. coli* enzyme in this region. The pH optimum for the *S. marcescens* enzyme was about pH 6.8 and 7.0 for the *E. coli* enzyme.

FIG. 4. Ultracentrifugal analysis of *L*-asparaginase (5 mg/ml) in 0.01 M sodium phosphate buffer (pH 6.9). The photographs were taken 24 min (A) and 72 min (B) after attaining a speed of 56,100 rev/min. Sedimentation is from left to right. The upper schlieren trace is *Escherichia coli* asparaginase, and the lower trace is *Serratia marcescens* asparaginase. Both enzymes had a specific activity of 200 IU per mg of protein.

FIG. 5. Effect of enzyme concentration on the sedimentation coefficients of *L*-asparaginase in 0.01 M sodium phosphate buffer (pH 6.9). The ordinate represents $S_{20,w}$ corrected for solvent viscosity and temperature.

Kinetic studies. Lineweaver-Burk plots of *S. marcescens* enzyme are shown in Fig. 7. An apparent K_m of 1.0×10^{-4} M was obtained at two different enzyme concentrations. Under the same

FIG. 6. Effects of pH on the activity of *L*-asparaginase from *Escherichia coli* and *Serratia marcescens* at 37 C. Incubations were conducted in excess substrate (10 μ moles/ml) for 15 min; all mixtures were adjusted to isoionic conditions ($\Gamma/2 = 0.15$) with the addition of NaCl. The following buffers were used: $\bullet$, pH 3.6 to 5.6, sodium acetate-acetic acid; Δ , pH 5.6 to 7.8, sodium phosphate; $\circ$, pH 7.4 to 9.0, Tris-hydrochloride; $\square$, pH 9.0 to 10.0, glycine-NaOH; $\blacksquare$, pH 9.2 to 10.7, carbonate-bicarbonate.

conditions, the K_m for the *E. coli* enzyme was 1.77×10^{-4} M (Fig. 7). Similar experiments were conducted with mouse serum in the reaction mixture in attempts to simulate physiological environments. No change in K_m was observed.

FIG. 7. Lineweaver-Burk plots of L-asparaginase. Velocity is expressed as micromoles of ammonia released per minute at pH 7.4 and 37 C as described under NADH-dependent coupled assay. *S* represents L-asparagine in moles per liter. The data show the results from experiments involving two different enzyme concentrations. A, *Serratia marcescens* enzyme; B, *Escherichia coli* enzyme.

Tumor inhibition by L-asparaginase. The comparative tumor inhibition of L-asparaginase from *E. coli* and *S. marcescens* is shown in Tables 2 and 3. Enzyme preparations of about the same specific activity were injected intraperitoneally in a single dose 6 days after tumor implantation. Tumors grew rapidly in animals not receiving L-asparaginase, and these mice usually died within 1 month. The *S. marcescens* enzyme at a dose of 2.5 IU produced a marked decrease in tumor size in 4 days, whereas the *E. coli* enzyme showed little effect with the same dose. Table 2 shows the change in tumor diameter after treatment with L-asparaginase from *E. coli* and *S. marcescens*. At a dose of 2.5 IU, the *S. marcescens* enzyme was more inhibitory to tumor growth than the *E. coli* enzyme. However, at a dose of 3.3 IU, both enzymes were equally inhibitory to tumor growth. Tumor-bearing mice were observed 1 year after treatment with enzyme; 0.83 IU of *S. marcescens* enzyme resulted in complete regression of tumors, whereas over 2.5 units of *E. coli* enzyme were needed to achieve the same result (Table 3). The tumor inhibitory effect of L-

TABLE 2. Effects of L-asparaginase on tumor growth of the 6C3HED lymphoma in the C3H mouse

Asparaginase administered (IU/animal) ^a	Mean change in tumor size (mm)		
	<i>Escherichia coli</i> enzyme	<i>Serratia marcescens</i> enzyme	Control
0			+8.8 (±0.71)
0.83	+5.5 (±0.48) ^b	+4.6 (±0.40)	
1.7	+3.1 (±0.46)	+1.8 (±0.44)	
2.5	+0.28 (±0.36)	-2.1 (±0.63)	
3.3	-4.0 (±0.24)	-4.1 (±0.42)	

^a Mice were given a single intraperitoneal injection of enzyme 5 days after tumor implantation. Each group contained 12 mice.

^b Values in parentheses are standard errors.

TABLE 3. Relative amounts of L-asparaginase from *Escherichia coli* and *Serratia marcescens* required for complete regression of the 6C3HED lymphoma in C3H mice

L-Asparaginase administered (IU/animal) ^a	Fraction of animals with complete tumor regression		
	<i>E. coli</i> enzyme	<i>S. marcescens</i> enzyme	Control
0.83	1/12	12/12	
1.7	0/12	12/12	
2.5	0/12	12/12	
3.3	5/8	8/8	
5.0	8/8	8/8	
None			0/8

^a Mice were given a single intraperitoneal injection of enzyme 5 days after tumor implantation. All mice were kept under observation for 1 year after tumor implantation.

asparaginase from *E. coli* and *S. marcescens* was independent of the specific activity of the enzyme preparations, provided that they were not toxic to the animals.

Blood clearance of L-asparaginase. The clearance of injected asparaginase from the blood of C3H mice before tumor implantation was measured. The *E. coli* enzyme was cleared more rapidly than the *S. marcescens* enzyme, the half-life of the former being 2 to 4 hr and the half-life of the latter being 6 to 8 hr (Fig. 8). When the above experiment was repeated in mice bearing the 6C3HED lymphoma, the blood clearance rate of injected L-asparaginase was sharply decreased to a half-life of 26 to 29 hr for both enzyme preparations. The maximum half-life of the *S. marcescens* enzyme was attained in 24 hr after tumor implantation, whereas the *E. coli* enzyme required 48 hr to reach a similar value. Since other researchers (24, 29) showed the presence of a virus in many transplantable murine tumors

FIG. 8. Blood clearance rates of L-asparaginase from *Serratia marcescens* and *Escherichia coli* after intraperitoneal injection into normal C3H mice. Each animal received a single injection of 74 IU of enzyme at zero time. Blood was taken from the orbital plexus and assayed for L-asparaginase.

and this virus caused an impairment of the blood clearance system, the blood levels of lactic dehydrogenase were measured before and after tumor implantation. Beginning at a normal value of 400 lactic dehydrogenase units/ml of blood, there was a threefold rise in blood level of lactic dehydrogenase within 48 hr after tumor implantation,

indicating the presence of infection by lactic dehydrogenase-elevating virus. A preparation of the partially purified virus containing 10^7 ID₅₀ per ml was injected into C3H mice with a resulting increase in blood level of lactic dehydrogenase similar to that found in the tumor-bearing mice. The blood clearance rate of injected L-asparaginase was measured in mice after infection with the virus; these animals bore no tumors. In these mice, the half-life of the enzyme from *S. marcescens* and *E. coli* manifested values similar to those found in tumor-bearing mice. These results indicate that the impairment of blood clearance of L-asparaginase in tumor-bearing mice was due to the presence of the virus in the transplanted tumor tissue which resulted in a viremia in the host, as suggested by other researches.

Immunogenicity of L-asparaginase. Preparations with a specific activity of 200 IU or greater manifested a single arc upon immunoelectrophoresis of the enzyme from *E. coli* and *S. marcescens* (Fig. 9).

Antibodies against enzymes can form insoluble antigen-antibody complexes which may or may not retain the catalytic activity of the enzyme. We found that this activity was retained in the case of L-asparaginase from *E. coli* and *S. marcescens*. We then determined which arcs on the immunoelectrophoresis slides contained L-asparaginase activity in the following manner. After electrophoresis, slides were washed with 0.01 M Veronal buffer (pH 8.6) for 24 to 48 hr at 6 C

FIG. 9. Immunoelectrophoretic comparison of L-asparaginase from *Serratia marcescens* and *Escherichia coli*. In the left well is *E. coli* asparaginase with a specific activity of 300 IU/mg of protein. In the right well is *S. marcescens* enzyme with a specific activity of 250 IU/mg of protein. After electrophoresis, antiserum was added to the center trough: A, antiserum against *S. marcescens* enzyme; B, antiserum against *E. coli* enzyme.

with multiple changes of buffer to remove all unprecipitated protein. The slides were then dried at 45 C for 4 to 6 hr, flooded with a solution containing 40 μ M asparagine/ml in 0.05 M Tris-hydrochloride buffer (pH 7.4), and incubated at 37 C for 5 to 20 min. The fluid was drained from the slide, and Nessler's reagent was added. Any arc containing active enzyme developed a yellow color. For instance, in Fig. 9A, two arcs can be seen in the *E. coli* preparation, but only the arc closer to the trough contained catalytically active enzyme according to the above test.

Ouchterlony gel diffusion of L-asparaginase from *E. coli* and *S. marcescens* against homologous and heterologous antiserum showed cross-reactivity between the two enzyme preparations. The precipitin bands of the two enzymes and antiserum against *S. marcescens* enzyme fused and formed a spur toward the *E. coli* enzyme, showing that the *E. coli* enzyme reacts with antiserum against the *S. marcescens* enzyme. This was also borne out in the slide immunoelectrophoresis. The reaction of *S. marcescens* enzyme with antiserum against *E. coli* enzyme was not visible on the Ouchterlony plates.

DISCUSSION

Campbell et al. (5), Roberts et al. (31) and Schwartz et al. (34) provided evidence that L-asparaginase from *E. coli* existed in two forms, one tumor inhibitory (EC-2) and the other inactive (EC-1) against mouse tumors. *E. coli* K-12 also contains two asparaginases, only one of which is tumor inhibitory (7). The strain of *S. marcescens* used in the present study manifested only one form of the enzyme, although extensive attempts have yet to be made to determine the existence of multiple forms of the enzyme. The purification procedures described herein yielded a preparation of L-asparaginase which was homogeneous according to the following criteria: (i) migration of a single symmetrical peak during boundary sedimentation velocity studies; (ii) the appearance of a single arc upon immunoelectrophoresis, and (iii) a single band in analytical polyacrylamide gel electrophoresis. The latter band was obtained only after the purified preparations were pretreated with SDS to prevent aggregate formation.

Attempts have been made to correlate the antitumor properties of L-asparaginase with certain of its physical properties. For instance, the enzyme from different sources may vary in substrate specificity. The enzyme from guinea pig serum does not hydrolyze glutamine, unlike the enzyme from bacterial sources which may contain about 5% glutaminase activity. The preparations of L-asparaginase from *E. coli* and *S. mar-*

cescens used in this work contained about 2 to 5% glutaminase activity. The pH activity curves of the two preparations were similar, whereas the K_m of the *E. coli* enzyme was slightly higher than the *S. marcescens* enzyme. However, this does not explain the increased antitumor activity of the *S. marcescens* enzyme in view of the observation of Ohnuma et al. (25) on L-asparaginase from guinea pig serum and chicken liver. They found that both enzymes had similar K_m values, although the chicken liver enzyme was relatively ineffective as an antitumor agent.

The tumor inhibitory property of L-asparaginase is dependent on its rate of clearance from the circulatory system after injection. The enzyme from guinea pig serum had a blood half-life of 19 hr compared to less than 1 hr for the yeast enzyme. The molecular weight of the former was reported as 133,000 by Yellin and Wriston (40), and the latter was given as about 800,000 by Broome (4). The yeast enzyme showed no inhibition of tumor growth, perhaps because it was cleared so rapidly that the blood asparagine level could not be sufficiently depleted (4). We found that the enzyme from *E. coli* and *S. marcescens* had the same sedimentation constant of 7.6S, indicating similar apparent molecular weights. Thus, the molecular weight alone does not determine clearance rate of the enzyme. Ohnuma et al. (25) determined the molecular weights of guinea pig serum enzyme and chicken liver enzyme as 210,000 and 306,000, respectively. They concluded that this difference could not account for the poor tumor inhibition of the chicken enzyme.

Kirschbaum, Wriston, and Ratych (17) reported that *E. coli* enzyme formed aggregates with molecular weights up to 255,000 during centrifugation of an enzyme solution containing 10 mg/ml. This transitory effect occurred 30 min after dissolution, and it was absent 3 hr later. We did not observe this phenomenon with similar preparations of *E. coli* enzyme analyzed 3 hr after dissolution. The existence of aggregates of L-asparaginase in vivo could possibly influence the clearance rate from the blood and thereby affect the tumor inhibitory property of the enzyme; however, this study has not yet been conducted.

It is clear that the increased blood half-life of the two enzymes in mice infected with the virus as well as tumor-bearing mice (also virus infected) in our experiments was due to a viremia. We observed the characteristic elevation of lactic dehydrogenase in the blood after tumor implantation (28, 29). No clear explanation is yet available for the difference in blood half-life values of the enzyme from *E. coli* and *S. marcescens* in

mice not infected with the lactic dehydrogenase virus.

Antibodies against the *E. coli* enzyme and the *S. marcescens* enzyme did not eliminate catalytic activity of either enzyme when it was complexed in a precipitin arc after immunoelectrophoresis. This may indicate different locations on the molecule of catalytic and antibody binding sites. Antibodies against homologous antigen showed differing degrees and nature of cross-reaction with the heterologous L-asparaginase, both without abolishment of catalytic activity. The one-sided cross-reaction of *S. marcescens* enzyme with antiserum against *E. coli* enzyme deserves further study. One may explain these results by viewing this system as a highly dissociated binding phenomenon between cross-reacting *S. marcescens* enzyme and antiserum against *E. coli* enzyme. This could be envisioned if the *Serratia* and *E. coli* enzymes shared certain identical or closely related antigenic sites of the enzyme surface, except that the tertiary structure was folded in a slightly different manner such that the site on the *Serratia* enzyme would be recognized by antibody to *E. coli* enzyme but the latter would not form a tight union with the *Serratia* antigen. Also, if the *Serratia* enzyme shared only minor reactive groups with the *E. coli* enzyme and had certain dominant antigenic groupings masked which determined the specificity of the homologous reaction, the heterologous union could be expected to be relatively weak and highly dissociated. Thus, the differences between the homologous and heterologous cross-reactions would seem to be due to the firm union of dominant antigenic and antibody groups in the first instance, and to the loose, highly dissociated union resulting from the reaction of minor or altered major groups in the latter case. The close similarity in physical and chemical properties of the *E. coli* and *S. marcescens* enzyme as described here does not point to any clear explanation for the increased inhibition of tumor growth shown by the *S. marcescens* enzyme.

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