INSOLUBILIZATION OF L-ASPARAGINASE BY COVALENT ATTACHMENT TO NYLON TUBING[1]

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L-asparaginase has been insolubilized by covalent attachment to partially hydrolyzed nylon tubing via the bifunctional reagent glutaraldehyde. The Km of the insolubilized enzyme decreases from 3 mM to 0.67 mM L-asparagine with increasing flow rate. The pH optimum of the enzyme is unchanged, although the range of optimal activity is somewhat restricted by insolubilization. Thermal stability of the enzyme is greatly enhanced by insolubilization. The asparaginase-nylon tubing is effective in clearing L-asparagine from human blood and use of the derivative in the asparaginase therapy of acute lymphocytic leukemia is suggested.

The useful role of the enzyme L-asparaginase (L-asparagine amino-hydrolase, E.C. 3.5.1.1) in the therapy of acute lymphocytic leukemia is well-known and has been the subject of several recent reviews (1, 2,3). As a foreign protein, Escherichia coli asparaginase is immunogenic in humans and production of antibodies to the drug has undesirable consequences. Hypersensitivity phenomena ranging from mild allergic reactions to anaphylactic shock have been reported in 5-20% of patients treated (3). In addition, production of antibody to the enzyme can result in loss of effectiveness of the drug due to shortened half-life in the plasma (4). One solution to these problems would be to have at hand several immunologically distinct tumor-inhibitory asparaginases. Alternatively, the enzyme could be administered in such a way as to render it non-immunogenic. Chang (5) accomplished this by enclosing asparaginase in semipermeable microcapsules into which asparagine could diffuse

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and be acted upon by the encapsulated enzyme. The microcapsules, when injected into the peritoneal cavity, were effective in inhibiting the growth of an implanted lymphosarcoma of mice. Injection of the enzyme could be avoided by the use of an extracorporeal shunt with asparaginase activity through which blood could flow and be cleared of asparagine. This paper describes the covalent attachment of asparagine to partially hydrolyzed nylon tubing and discusses some of the properties of the insoluble asparaginase derivative.

Materials and Methods

"Lyovac" L-asparaginase (Lot C-8378) was the generous gift of Merck, Sharp, and Dohme Research Laboratories. Type 6 nylon tubing (0.1 cm internal diameter) was obtained from John Tullis & Son, Ltd. (Alloa, Tullibody, Scotland). NADH was purchased from P-L Biochemicals, Inc. L-asparagine was obtained from Mann Research Laboratories. Malate dehydrogenase (pig heart), L-glutamate oxaloacetate transaminase (Type I, pig heart), and α-ketoglutarate (monosodium salt) were purchased from Sigma Chemical Company. Nessler's reagent was from Anderson Laboratories and glutaraldehyde (25% in H_2O) was purchased from J.T. Baker Chemical Co. A Buchler polystaltic pump with "tygon" pumping tubes was used for perfusion of the nylon tubing. Human blood was obtained by venipuncture and collected in a heparinized bottle.

The method used for attachment of the asparaginase was a modification of the technique of Sundaram and Hornby (6). A coil of nylon tubing (2 meters long) was placed in a 35° water bath and its inner surface partially hydrolyzed by pumping through the tubing 4.5 M HCl for 15 minutes at a flow rate of 5 ml/min. Hydrolysis was arrested by pumping water through the tubing for 15 minutes. The coil was then transferred to an ice bath and rinsed with 0.2 M sodium bicarbonate buffer, pH 9.0, for 2 minutes. The tubing was then perfused with a 5%

(v/v) solution of glutaraldehyde in 0.2 M sodium bicarbonate, pH 9.4, for 15 minutes at a flow rate of 2 ml/min. After the tubing was rinsed with 0.05 M sodium phosphate buffer, pH 8.0, it was perfused for 1.5 hours with 10 ml of a dialyzed solution of "Lyovac" asparaginase (22 mg protein, 7530 enzyme units) in the same buffer. Uncoupled enzyme was removed by perfusion of the tubing at 0° with 0.1 M sodium bicarbonate, 0.1 M sodium chloride, and finally with water for 10 minutes each at a flow rate of 5 ml/min. The tubing was then cut into two equal portions and stored in water, one section at 4° and the other at room temperature.

The asparaginase activity of the tubing was determined by measuring either the ammonia or the aspartate formed when solutions of L-asparagine in 0.1 M Tris-HCl, pH 8.0 were passed through the tubing at predetermined flow rates. Ammonia was determined by Nesslerization according to the method of Mashburn and Wriston (7). L-aspartate and L-asparagine were determined by the NADH-dependent coupled assay described by Cooney et al. (8).

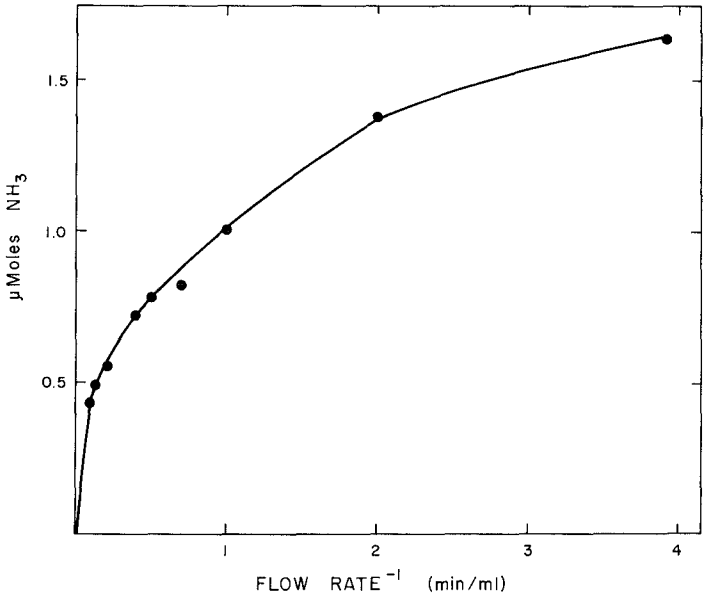

Figure 1. The effect of flow rate on production of ammonia. A 1 meter coil of asparaginase-nylon tubing was placed in a 37° water bath and perfused at predetermined flow rates with 0.01 M L-asparagine in 0.05 M Tris-HCl, pH 8.6. Ammonia was determined by Nesslerization (7).

Results and Discussion

L-asparaginase was effectively coupled to partially hydrolyzed nylon tubing by the glutaraldehyde crosslinking procedure described above. The effect of flow rate on the production of ammonia by the asparaginase-nylon tubing is illustrated in Figure 1. The tubing was found to have a specific activity of 3.18 μmoles aspartate/min/meter at a flow rate of 1.8 ml/minute. The asparaginase-nylon tubing was very stable upon storage. No loss of activity could be detected after two months of storage in water at 4°. A section of tubing stored in water at room temperature retained 56% of the initial activity after the same period.

Kinetic data indicate an increase in apparent Km upon insolubilization. Soluble E. coli asparaginase has a Km of 1.3×10^{-5} M for L-asparagine (9). The asparaginase-nylon tubing gave a value of 3×10^{-3} M

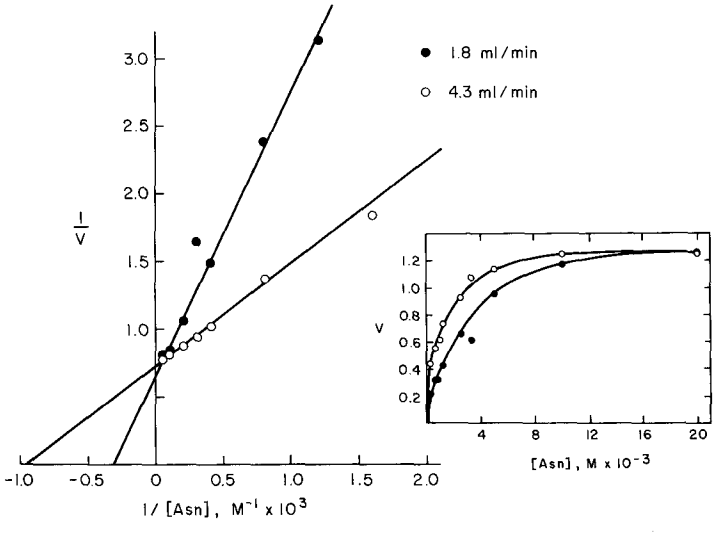

Figure 2. Lineweaver-Burk plots for asparaginase-nylon tubing at two flow rates. Inset: Substrate saturation profile of asparagine-nylon tubing at two flow rates. An 0.4 meter section of asparaginase-nylon tubing was placed in a 37° water bath and perfused with various concentrations of L-asparagine in 0.1 M Tris-HCl, pH 8.0. Liberated aspartate was determined spectrophotometrically according to Cooney et al. (6). Velocity (v) is defined as μmoles aspartate formed per minute.

when a flow rate of 1.8 ml/minute was used. This loss of affinity for substrate is probably largely a result of a change in conformation of the enzyme due to chemical modification during the coupling process. However, charge-charge interactions between substrate and the support might also be expected to contribute to the apparent increase in Km. At pH 8.0, both the partially hydrolyzed nylon and L-asparagine possess net negative charge, and the like charges would have the effect of lowering the relative substrate concentration in the neighborhood of the enzyme (10).

Kinetic analysis also revealed a dependence of Km on the flow rate. Figure 2 illustrates the decrease in Km observed with increased flow rates. At a flow rate of 4.3 ml/min, the apparent Km was 0.67 mM L-asparagine. Flow rates of 6.0 ml/min and 8.3 ml/min gave substrate saturation curves and double reciprocal plots similar to those shown for the 4.3 ml/min rate. This asymptotic decrease in Km with increasing flow rate suggests diffusion-limited transport of substrate into the microenvironment of the enzyme. A similar phenomenon has been described for the hydrolysis of benzoyl-L-arginine ethyl ester in packed columns of a carboxymethyl cellulose-ficin derivative (11).

Other properties of the enzyme are changed upon insolubilization. A comparison of the pH profiles of soluble and covalently-coupled asparaginase is shown in Figure 3. Asparaginase-nylon tubing shows a more restricted range of activity although optimum activity is obtained at pH 8.0 with both forms of the enzyme. As shown in Figure 4 the insoluble asparaginase is considerably more resistant to thermal inactivation than is the enzyme free in solution.

In order to determine the effectiveness of the asparaginase-nylon tubing under physiological conditions, asparagine and aspartate levels were determined in fresh whole human blood which had been passed through the tubing and compared with the levels of the amino acids in untreated

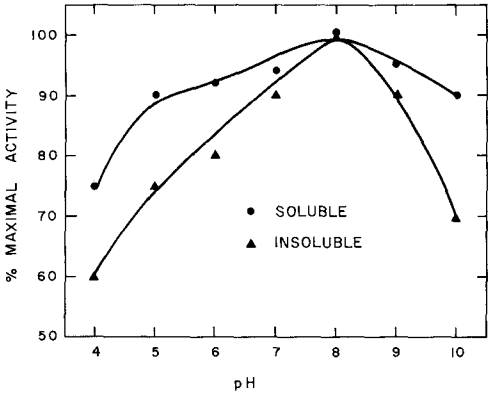

Figure 3. The pH activity profiles of asparaginase-nylon tubing and free enzyme. Substrate solutions of 0.01 M L-asparagine in the following 0.05 M buffers were used in each case: pH 4-6, sodium acetate; pH 6-8, sodium phosphate; pH 8-10, sodium borate. Soluble L-asparaginase was incubated in substrate for 10 minutes at 37° C, at which time the reaction was stopped with 1.5 M trichloracetic acid and ammonia determined by Nesslerization (7). Asparaginase-nylon tubing (1 meter) was placed in a 37° water bath and perfused with substrate at a flow rate of 2.0 ml/min. Liberated aspartate was determined spectrophotometrically (6).

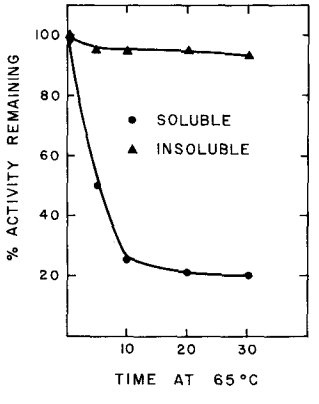

Figure 4. The effect of temperature on the stability of asparaginase-nylon tubing and free asparaginase. The data for the free enzyme were obtained as follows: 0.2 ml aliquots of asparaginase in 0.05 M Tris-HCl, pH 8.6, were placed in a 65° water bath at 0 time. Aliquots were removed at specified intervals and placed in ice. Residual activity was determined as previously described (7). The data for the asparaginase-nylon tubing was obtained by incubating an 0.12 meter section of tubing in a 65° water bath while perfusing it with water at the same temperature at a flow rate of 3.3 ml/min. At the specified intervals, 2.5 ml of 0.01 M L-asparaginase in 0.1 M Tris-HCl, pH 8.0, was passed through the tubing. Aspartate in the effluent was determined as previously described.

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blood. As can be seen from Table 1, plasma L-asparagine was reduced

Table 1

THE CLEARANCE OF L-ASPARAGINE FROM WHOLE HUMAN BLOOD

BY ASPARAGINASE-NYLON TUBING

	Plasma (nmoles/ml)		Erythrocytes (nmoles/ml)	
	L-Aspartic acid	L-Asparagine	L-Aspartic acid	L-Asparagine
Untreated	8	48	313	104
Treated	29	29	353	108

Fresh whole human blood was collected by venipuncture and drawn into a heparinized bottle. "Treated" blood was passed through a 1 meter length of asparaginase-nylon tubing at 37° and a flow rate of 2 ml/min. Untreated blood was not passed through the tubing. Plasma and erythrocytes were separated by centrifugation at 1,000 Xg. After a 1:2.5 dilution in H_2O of the packed erythrocytes, the samples were processed and amino acid levels determined as described by Cooney et al. (8).

40% during a single rapid passage of whole blood through the tubing. More efficient clearance of asparagine could be obtained by preparing asparagine-nylon tubing derivatives with greater activity per unit length or by slower circulation of blood through the tubing.

This paper shows that tubing with asparaginase activity can be prepared by covalent attachment of the enzyme to the partially hydrolyzed interior surface of nylon tubing. The insolubilized asparaginase is considerably more stable than is the enzyme free in solution. While the affinity of the enzyme for substrate is decreased, as indicated by an increase in Km from 10^{-5} M to 10^{-3} M, the observed Km of the asparaginase-nylon tubing depends on flow rate. The tubing does possess sufficient asparaginase activity at low asparagine concentrations to be effective under physiological conditions. In vivo experiments to investigate the applicability of extracorporeal shunts of asparaginase-nylon tubing to the chemotherapy of leukemia are planned.

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