Biochemical Pharmacology, Vol. 24, pp. 503–515. Pergamon Press, 1975. Printed in Great Britain.

BIOCHEMICAL AND PHARMACOLOGIC PROPERTIES OF L-ASPARAGINASE BONDED TO DACRON VASCULAR PROSTHESES

DAVID A. COONEY, HOWARD H. WEETALL and EDWIN LONG

National Cancer Institute, National Institutes of Health, Bethesda, Md. 20014 (D.A.C.), Corning Glass Co., Corning, N.Y. 14830 (H.H.W.) and Pennsylvania Hospital, Philadelphia, Pa. 19107 (E.L.), U.S.A.

(*Received* 17 *December* 1973; *accepted* 12 *July* 1974)

Abstract—L-Asparaginase has been covalently coupled to Dacron vascular prostheses, and the hydrolytic and biochemical properties of the resultant "enzyme grafts" have been explored *in vitro* and *in vivo*. Amidohydrolysis was shown to be dependent on the rate of flow, character of the perfusate, temperature, concentration of substrate and pH. Placements in the inferior vena cava of dogs significantly perturbed the homeostasis of L-asparagine for periods of over 1 week. Conversely, implantations in the iliac artery failed to depress the level of circulating L-asparagine. Accumulation of protein on the intimal face of the graft impaired the accessibility of enzyme to its circulating substrate. In mice bearing ascitic tumors sensitive to L-asparaginase, these enzyme grafts produced no increase in survival time. Normal mice responded to intraperitoneal implantations of circlets of Dacron-L-asparaginase with the elaboration of humoral antibodies to the enzyme. *In vitro*, anti-L-asparaginase antibodies diminished the enzymatic efficiency of the grafts. Evidence is presented that the covalent bond between the polymer and the protein is gradually ruptured, and that the hydrolytically active L-asparaginase so released is responsible for the immunogenicity of these prostheses.

Antibodies to the oncolytic enzyme L-asparaginase are known to arise in lower animals and in man given parenteral injections of the agent [1]. Moreover, immunity to the enzyme can compromise its therapeutic activity [2, 3]. In an attempt to circumvent this immunogenicity, as well as to produce a sustained dosage form of the enzyme, L-asparaginase has been covalently coupled to Dacron vascular prostheses. Such a stable bond of protein to polymer should effectively prevent the enzyme-antigen from entering antibody-forming cells. It is the purpose of the present communication to describe the biochemical and pharmacologic properties of these L-asparaginase grafts *in vitro* and *in vivo*.

MATERIALS AND METHODS

L-Asparaginase from *Escherichia coli* (EC-2) was a donation of Merck, Sharp & Dohme to the National Cancer Institute of the National Institutes of Health, U.S.A. The techniques of coupling the enzyme to Dacron prostheses have been described [4]. Heparin was introduced into the prosthesis by thermal impregnation [5]. Measurement of L-asparaginase was made by enzymatic spectrophotometric techniques [6]. When low levels of amido-hydrolase ($< 1 \times 10^{-3}$ i.u./ml) were being monitored, UL-[^{14}C]L-asparagine was used as the substrate. The catalytic decomposition of L-DONV (5-diazo-4-oxo-L-norvaline) was monitored at 274 nm, using a molar extinction coefficient of 11×10^3 [7]. [^{14}C]L-DONV was provided by Dr. H. Wood of the National Cancer Institute of the National Institutes of Health, U.S.A. D-DONV and L-asparaginase inactivated (99 per cent) with L-DONV were generous gifts of Dr. R. E. Handschumacher of Yale University School of Medicine, New Haven, Conn., U.S.A. Ammonia released by the hydrolysis of D-asparagine, D-glutamine and L-glutamine was measured with L-glutamate dehydrogenase and NADH [8]. Hydroxamate formation was detected with a ferric chloride reagent as described by Jayaram *et al.* [9]. Antisera were raised in mice by the repeated intramuscular injection of EC-2 in complete Freund's adjuvant. Antibodies to L-asparaginase were measured by passive hemagglutination according to the methodology of Peterson *et al.* [1]. The clearance of 4-[^{14}C]L-asparagine was monitored by an enzymatic radiometric technique specific for L-aspartic acid and L-asparagine; the details of this technique have been presented in full elsewhere [10]. Proteins were measured by the method of Lowry *et al.* [11]. Venous and arterial placements of the prostheses in dogs were made according to standard surgical techniques.

RESULTS

Inasmuch as the covalent conjugation of L-asparaginase to Dacron might be expected to change the catalytic properties of the enzyme, a series of systematic studies was undertaken comparing the response of the free and bound enzyme to a variety of modifiers.

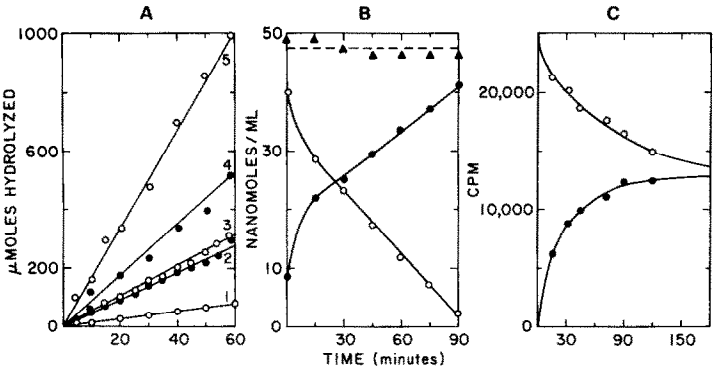

Fig. 1. (A) Influence of substrate concentration and flow-rate on the hydrolysis of L-asparagine by L-asparaginase covalently coupled to a Dacron prosthesis. Line 1: 3 l. L-asparagine, $1{\cdot}5 \times 10^{-4}$ M in 0·05 M Tris-HCl, pH 8·3, 37°, was pumped through a 7-cm length of Dacron tubing bearing 20 i.u. L-asparaginase at a flow-rate of 0·2 l/min. The substrate recirculated for the duration of the experiment. Line 2: conditions same as in Line 1 except that flow-rate was 0·6 l/min. Line 3: conditions same as in Line 1 except that flow-rate was 0·7 l/min. Line 4: conditions same as in Line 1 except that concn of L-asparagine was $1{\cdot}5 \times 10^{-3}$ M and flow-rate 0·5 l/min. Line 5: conditions same as in Line 1 except that concn of L-asparagine was $1{\cdot}5 \times 10^{-3}$ M and flow-rate 0·7 l/min.

(B) Time-course of hydrolysis of the L-asparagine in human plasma by a Dacron prosthesis to which L-asparaginase was covalently bound. One liter human plasma at 37° at a flow-rate of 2 l/min was pumped through a Dacron prosthesis bearing 10 i.u. L-asparaginase. At 15-min intervals, 2-ml samples were removed, boiled for 20 min, and centrifuged at 104,000 *g* for 30 min. The concns of L-aspartic acid and L-asparagine were measured spectrophotometrically in the supernatants [6]. Key: (○) L-asparagine; (●) L-aspartic acid; and (▲) L-asparagine and L-aspartic acid.

(C) Hydrolysis of L-asparagine in whole human blood by L-asparaginase covalently coupled to Dacron tubing. L-Asparaginase (10 i.u.) was covalently coupled to a Dacron prosthesis as described in Methods. Human blood (200 ml) was anticoagulated with 1000 units heparin; then 10 μCi 4-[^{14}C]L-asparagine was added. The blood was warmed at 37° and pumped through the tubing at a flow-rate of 100 ml/min. Samples of whole blood were removed at the times indicated in the figure and plasma was separated at 4°. The plasmatic L-asparagine, L-aspartic acid, 4-[^{14}C]L-asparagine and 4-[^{14}C]L-aspartic acid were measured as described in Methods. The concn of L-aspartic acid in the 0-time sample was 5 nmoles/ml, and the concn of L-asparagine at the same time was 35 nmoles/ml. At 90 min, the concn of L-aspartic acid had risen to 40 nmoles/ml, and the concn of L-asparagine had fallen to 15 nmoles/ml. Since some hemolysis transpired during the course of the perfusion, the sum of the dicarboxylic amino acids, L-aspartic acid plus L-asparagine, deviated somewhat from theory in the final sample. The open circles (○) represent 4-[^{14}C]L-asparagine; the closed circles (●) represent 4-[^{14}C]L-aspartic acid. During the course of the perfusion, a portion of the intimal surface of the graft near the outflow became coated with fibrinous material.

Influence of the rate of flow of substrate on the hydrolytic activity of covalently bound L-*asparaginase.* The velocity of hydrolysis of L-asparagine by the enzyme-bearing graft was a direct function of the rate of flow of the substrate through the graft (Fig. 1A). Rapid passage of the substrate over the graft serves to expose the catalytic site to a continuous flow of L-asparagine as well as to remove ammonia and L-aspartic acid, both of which are relatively powerful product inhibitors of the enzyme [12, 13]. It should be noted from Fig. 1 that hydrolysis proceeded linearly for over an hr at all flow rates when pure solutions of L-asparagine were used. However, when plasma was pumped through a Dacron graft (Fig. 1B) at a velocity of 2 l/min, the initial rapid rate of hydrolysis was maintained only for ~ 12 min, whereafter a second slower rate of hydrolysis supervened and was maintained for the duration of the experiment. With blood as the perfusate, a similar phenomenon was observed (Fig. 1C). This early deviation from linearity probably results from two factors: (1) as will be shown later, the covalently coupled enzyme is undersaturated at the concentration of L-asparagine present in human plasma (0·05 mM), and (2) a film of protein or fibrin was found to have coated the graft, thereby impairing the diffusion of substrate and products into and from the catalytic site.

Alterations of the Michaelis constants of L-*asparaginase toward its prototypical substrates after covalent conjugation to Dacron.* It has been observed frequently that enzymes coupled to inert supports exhibit altered affinity constants for their substrates, or altered maximal velocities at saturation [14]. The Michaelis constants of free and bound L-asparaginase, therefore, were determined with L-asparagine, L-glutamine, β-cyano-L-alanine and L-DONV, four compounds which are prototypical substrates for the enzyme. In addition, the kinetics of decomposition of L-aspartyl-β-hydroxamate and DL-aspartyl-β-hydrazide by the free and

Table 1. K_m and V_{max} values of L-asparaginase in solution and covalently coupled to Dacron*

Substrate	Type of hydrolysis	Free L-asparaginase		Bound L-asparaginase	
		K_m (M)	Relative V_{max}	K_m (M)	Relative V_{max}
L-Asparagine	β-Amidohydrolysis	$1{\cdot}2 \times 10^{-5}$	100·0	$2{\cdot}0 \times 10^{-3}$	100·0
L-Glutamine	γ-Amidohydrolysis	$2{\cdot}7 \times 10^{-3}$	2·6	$1{\cdot}0 \times 10^{-2}$	9·0
β-Cyano-L-alanine	Nitrilase	$5{\cdot}4 \times 10^{-3}$	3·4	$1{\cdot}1 \times 10^{-2}$	23·0
5-Diazo-4-oxo-L-norvaline	"Diazotase"	$9{\cdot}5 \times 10^{-6}$	1·9	$3{\cdot}0 \times 10^{-4}$	1·3
DL-Aspartyl-β-hydrazide	β-Hydrazide hydrolysis	$1{\cdot}0 \times 10^{-3}$	1·0	$2{\cdot}0 \times 10^{-3}$	4·0
L-Aspartyl-β-hydroxamate	β-Hydroxamate hydrolysis	$5{\cdot}3 \times 10^{-4}$	8·0	$6{\cdot}3 \times 10^{-4}$	30·0
Hydroxylamine (plus L-asparagine)	Transfer	$5{\cdot}5 \times 10^{-2}$	7·7	$2{\cdot}0 \times 10^{-2}$	9·0
D-Asparagine	β-Amidohydrolysis	$4{\cdot}0 \times 10^{-2}$	4·8	$1{\cdot}7 \times 10^{-2}$	10·7

* In the case of the free enzyme: the hydrolysis of L-asparagine was measured at five concns of substrate between 1×10^{-4} and 1×10^{-3} M; L-aspartate production was quantitated spectrophotometrically [6]; the hydrolysis of D-asparagine and L-glutamine was measured at substrate concns between 1×10^{-3} and 1×10^{-2} M; ammoniagenesis was measured enzymatically [8]; the hydrolysis of β-cyano-L-alanine was measured at substrate concns ranging from 1×10^{-3} to 1×10^{-2} M and L-aspartic acid production was assessed enzymatically [6]; the hydrolysis of L-DONV was followed spectrophotometrically at 274 nm using an extinction coefficient of 11×10^3; the decomposition of DL-aspartyl-β-hydrazide and hydroxamate was measured at eight concns of substrate between 5×10^{-4} M and 5×10^{-2} M. (Both of these substrates give rise to products which are powerful inhibitors of both the free and bound enzyme, and no provision was made for the removal of these products here, so that the values given must be viewed as provisional.) The transfer reaction between L-asparagine and neutralized hydroxylamine was carried out using 0·01 M L-asparagine and concns of neutralized hydroxylamine varying from 0·1 to 0·001 M. L-Aspartyl-β-hydroxamate was quantitated with the acid ferric chloride reagent of Jayaram *et al.* [9]. In the case of the bound enzyme, the following concentrations were used: L-asparagine, 5×10^{-4} to 5×10^{-2} M; D-asparagine, 5×10^{-4} to 5×10^{-2} M; D-glutamine, 5×10^{-4} to 1×10^{-2} M; β-cyano-L-alanine, 1×10^{-4} to 1×10^{-2} M; DL-aspartyl-β-hydroxamate and hydrazide, 1×10^{-4} to 5×10^{-2} M; L-DONV, 5×10^{-5} to 5×10^{-4} M; the formation of products or consumption of substrates was followed in the same way as described above. In most cases, the assay was conducted in two stages: hydrolysis, followed by measurement of product. HCl was used to terminate the incubation step and was thereafter neutralized with equimolar NaOH. The decomposition of L-DONV, however, was followed directly in the thermostatted cell of the Beckman DBG recording spectrophotometer [7]. Times of incubation and unitage of enzyme were arranged so that no more than 20 per cent of the substrate was consumed by the close of the reaction. For the measurement of hydrolysis of the several substrates by bound L-asparaginase, separate circlets were cut in series from a Dacron prosthesis bearing L-asparaginase in covalent linkage so that each circlet produced ~0·5 i.u. of activity vs 0·01 L-asparagine in 0·05 M Tris-HCl buffer, pH 8·45. Incubations were carried out as described in the legend to Table 3, and terminated by removal of the circlet whereafter the substrate solutions were frozen until the time of assay.

bound enzyme were studied, as well as the synthesis of L-aspartyl-β-hydroxamate from L-asparagine and hydroxylamine. Table 1 presents the results of these studies: it can be seen that the affinity of the enzyme for its prime substrate, L-asparagine, has been reduced markedly by covalent attachment to the Dacron support. This depression in affinity is doubtless of therapeutic importance, inasmuch as amidohydrolases with K_m values ten times greater than the molarity of L-asparagine in physiologic fluids (~50 μM) have usually been found to be devoid of oncolytic potency [15].

Also depressed by the process of conjugation was the affinity of the bonded enzyme for L-glutamine, β-cyano-L-alanine and L-DONV. On the other hand, little change in the Michaelis constant of immobilized L-asparaginase was seen with DL-aspartyl-β-hydrazide or L-aspartyl-β-hydroxamate as substrates, and its affinity for D-asparagine and hydroxylamine even appeared to increase as a consequence of conjugation. In terms of the alteration in the maximal velocity reached by the bound enzyme under conditions of saturation, it is notable that this parameter increased, relative to the V_{max} with L-asparagine, for every substrate except L-DONV. While the increase in the relative velocity of hydrolysis of L-glutamine might be expected to confer on immobilized L-asparaginase the ability to deplete plasma of L-glutamine more effectively than the free species, it should be recalled that the bound enzyme is very markedly undersaturated *in vivo*, where the concentration of L-glutamine approximates 0·5 mM. Results touching on this point will be presented later, in connection with Fig. 7.

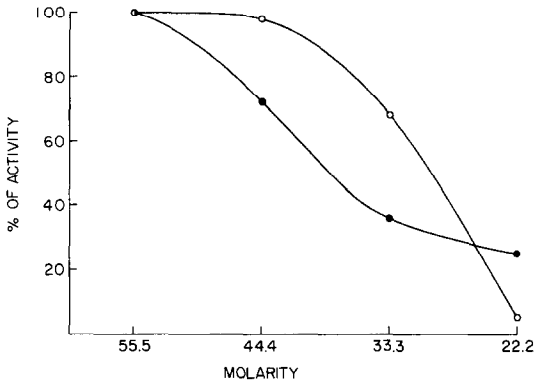

Fig. 2. Influence of hydropenia on the hydrolytic velocity of free and bound L-asparaginase. The molarity of water was reduced by the addition of an appropriate volume of dimethylsulfoxide (DMSO). In each of the substrate solutions, the final molarity of L-asparagine was 0·01 M and that of potassium phosphate was 0·1 M. At the highest concn of DMSO, a fine precipitate of substrate or buffer was deposited during the incubation; no correction for this effect has been made. In the case of the free enzyme, 0·1 i.u. L-asparaginase from *E. coli* was added to 5 ml of the substrate solution to inaugurate the reaction. Thirty min later, 500 μl of 1 N HCl was added, followed in 10 min by 500 μl of 1 N NaOH. The generation of L-aspartic acid was quantitated by an enzymatic spectrophotometric technique [6]. In no case did the concn of DMSO interfere with this assay. In the case of the bound enzyme, a section of Dacron bearing 1 i.u. L-asparaginase from *E. coli* was incubated in the appropriate substrate for 2 min at 37°, with agitation at the rate of 100 oscillations/min. The graft was then removed and reassayed in 0·01 M L-asparagine in 0·05 M Tris-HCl, pH 8·4. Any decrement in activity detected in this reassay vs the original activity was taken into account in the calculations. The closed symbol (●) stands for bound enzyme; the open symbol (○) stands for free enzyme.

Pursuant to these findings, a comparison also was made of the influence of the flow rate on the Michaelis constant of the bound enzyme for its principal substrate. In a still solution of L-asparagine, the K_m was 3×10^{-2} M, whereas, with agitation at the rate of 100 back-and-forth oscillations/min, the K_m fell by an order of magnitude to 3×10^{-3} M. The simplest interpretation of these results is that agitation prevents a zone of depleted substrate and accumulated product from forming on the surface of the prosthesis.

Since water is an essential reactant in the enzymatic as well as non-enzymatic decomposition of L-asparagine, the influence of alterations in the molarity of H_2O on amidohydrolysis by free and bound L-asparaginase was explored (Fig. 2). Dimethylsulfoxide (DMSO) was used to achieve a progressively hydropenic environment inasmuch as that solvent—at least at concentrations of 50% (v/v)—is known not to denature the free enzyme [7]. As the molarity of water was lowered below 44·4 M, the free enzyme became a progressively poorer hydrolase with only 5·5 per cent residual activity at a molarity of 22·2. The bound enzyme responded more promptly to slight decrements in the concentration of water, with a 28 per cent reduction in rate at a molarity of 44·4; but at a molarity of 22·2, it retained a percentage of its original activity far greater than the free enzyme. Qualitatively similar results were obtained when glycerol, dioxane and ethanol were used to lower the molarity of water. While these effects have been attributed to hydropenia, it is not impossible that the solvents were behaving as feeble inhibitors or denaturants of the bound enzyme. In fact, the two species were found not to be equally sensitive to inactivation by dimethylsulfoxide. Thus, 30 min of exposure to 20, 40 and 60% (v/v) solutions of DMSO in 0·1 M phosphate buffer, pH 7·4, produced 0, 2 and 11 per cent inactivation of L-asparaginase in solution, but 4, 20 and 31 per cent inactivation of the conjugated enzyme. If corrections are made for this inactivation by DMSO, the ability of bound L-asparaginase to function as a hydrolase in a hydropenic domain stands in even greater contrast to the performance of the free enzyme.

Stereospecificity of L-asparaginase coupled to Dacron. Free L-asparaginase from *E. coli* can decompose D- as well as L-asparagine, but the D-isomer is hydrolyzed at a rate 5–7 per cent of that seen with levorotatory asparagine [13]. This lack of absolute stereospecificity suggests that the configuration of the active site is sufficiently spacious to allow fixation of the primary amino

Table 2. Stereospecificity of free and conjugated L-asparaginase from *E. coli**

Substrate	Relative velocity of hydrolysis	
	Free enzyme	Bound enzyme
L-Asparagine	100·0	100·0
D-Asparagine	4·8	10·7
L-Glutamine	2·6	9·0
D-Glutamine	0·1	0·3
L-DONV	1·9	1·3
D-DONV	0·3	0·1

* Ammonia released by the hydrolysis of the amides at 37° was measured with L-glutamate dehydrogenase [8]. The decomposition of the diazoketones was monitored spectrophotometrically at 274 nm [7].

function regardless of its stereochemical configuration. Inasmuch as covalent fixation of L-asparaginase to the Dacron prosthesis can be expected to have involved carboxyl functions of the enzyme, and inasmuch as these same functions could be expected to be included in ionic interactions with the amino function of either D- or L-asparagine, it was felt to be important to characterize the reactivity of conjugated L-asparaginase toward a panel of stereoisomeric substrates. The results of this study are given in Table 2, where it can be seen that the enzyme has become a more efficient D-asparaginase as a consequence of its attachment to the Dacron support. Moreover, its D-glutaminase activity has been increased substantially. These results, which are in principle similar to those seen during the recovery of free L-asparaginase from exposure to elevated temperatures [16, 17], suggest that conjugation has distorted the center of catalysis to a configuration similar to that achieved by heat.

In order to investigate this change more thoroughly, the stereospecificity of the bound enzyme was explored at several non-denaturing temperatures. When the L- and D-asparaginase activity of the free enzyme was assessed at 2°, 24°, 37° and 56°, the ratio of the velocity with the L-isomer relative to that with D-isomer increased from 1·75 to 3·95 to 4·77 to 5·98. On the other hand, in the case of the bound species, this ratio fell from 28 to 9, 10·7 and 9·3, respectively, as the temperature was raised. Taken in conjunction with the finding of an augmented D-asparaginase activity of L-asparaginase coupled to Dacron, these results suggest that the active site of the bound enzyme is immobilized by the process of conjugation so that it cannot respond in a normal manner to thermal perturbations, i.e. that elevated temperatures cannot increase its D-asparaginase activity inasmuch as it is already at a maximum as a consequence of conjugation.

Response of bound L-asparaginase to pH changes. In order to uncover further differences brought about by the process of chemical conjugation, the pH optimum of the free and bound L-asparaginase versus L-asparagine was determined in 0·05 M phosphate buffers at 15 pH values between pH 6·1 and 8·9. In keeping with the findings of other workers, the free enzyme was found to exhibit a broad optimum between pH 7·6 and 8·9, with a blunt peak centering at pH 8·3; also the free enzyme was 30 and 60 per cent as active at pH 6·1 and 8·9, respectively, as at its optimum. Bound L-asparaginase also exhibited a broad optimum between pH 7·0 and 8·2, with a fairly sharp peak at pH 7·2. At pH 7·0 and 7·9, the hydrolytic velocity of the conjugated enzyme fell to 75 and 80 per cent, respectively, of the velocity detected at pH 7·2, while at pH 6·1 and 8·9, the activity was further depressed, but only to 65 and 68 per cent of that seen at the peak. Thus it would appear that the process of conjugation makes L-asparaginase a somewhat more efficient catalyst near neutrality and minimizes the depressive effect of extremes of pH on its catalytic velocity. For the reasons given above, the influence of pH on the hydrolysis of D-asparagine by free and bound L-asparaginase also was determined at 13 pH values between 5·5 and 9·0. In accordance with the results of Nakamura *et al.* [13], we observed that the pH optimum of free L-asparaginase for D-asparagine was ~7; at pH 5·5 and 9·0 there was a 33 per cent reduction in V_{max}. With conjugated L-asparaginase, the optimum was shifted to pH 6·9; at pH 5·5 and 9·0 the per cent reduction in velocity was identical to that observed with the free species.

Since ionizable groups are utilized in the chemical conjugation of protein to Dacron, it was likely that the net charge of L-asparaginase would be altered in the process of coupling. For obvious reasons, it has not been possible to examine this change directly. However, in order to gain an indirect insight into such an alteration, the K_m of free and bound L-asparaginase was measured at a wide variety of pH values. It was anticipated that the relative unavailability of dissociable functions in the bound enzyme would obscure its response to major shifts in the hydrogen ion concentration. This was found to be the case (Table 3). The Michaelis constant of free L-asparaginase for L-asparagine rose from $\sim 1{\cdot}6 \times 10^{-5}$ M at pH 6·3 through 9·0, to 2×10^{-4} M, at pH 4·6, and to $8{\cdot}0 \times 10^{-4}$ M at pH 3·8. This is a 50-fold alteration. By contrast, the K_m of the bound enzyme did not vary by more than 2- or 3-fold over a range of pH from 3·8 to 9·8.

Influence of ionic strength on the Michaelis constant of bonded L-asparaginase. Zaborsky [18] has summarized data which indicates that the ionic strength of the incubation medium can alter profoundly the apparent affinity of an insolubilized enzyme for its substrate. Experiments, therefore, were designed to examine the influence of a spectrum of ionic strengths on the apparent affinity of Dacron-bound L-asparaginase for its principal substrate L-asparagine. In 0·001 M, 0·01 M, 0·1 M and 1·0 M Tris-HCl, pH 8·4, the Michaelis constants of the enzyme were found to be 3·3, 4·0, 3·7 and $4{\cdot}1 \times 10^{-3}$ M respectively. These values are all 100 times higher than the K_m of the native enzyme. If the theorem of Zaborsky is correct, this change in affinity should be the result of repulsion from similarly

Table 3. Influence of pH on the Michaelis constant of free and bound L-asparaginase for L-asparagine*

pH	Michaelis constant ($\times 10^{-4}$ M)	
	Free L-asparaginase	Bound L-asparaginase
3·80	8·00	69
4·00	8·00	83
4·60	2·00	89
5·15	1·70	50
6·30	0·14	63
7·00	0·16	40
8·40	0·16	56
8·50	0·18	64
9·00	0·17	20
9·80	0·31	26

* Solutions of L-asparagine ranging from 1×10^{-4} M to 1×10^{-3} M in the studies with the free enzyme, and from 1×10^{-3} to 1×10^{-2} M in studies with the bound enzyme, were prepared in 0·05 M sodium citrate (pH 3·8 to 6·3) and 0·05 M Tris-HCl (pH 7·0 to 9·8). Five molar concns of substrate were used for the determination of each K_m. With the free enzyme, unitage of L-asparaginase sufficient to hydrolyze no more than 20 per cent of the substrate at the pH in question was added to 5 ml of the L-asparagine solutions at zero time. After 30 min at 37°, the reaction was stopped with 500 μl of 1 N HCl; after an additional 10 min at 37°, the HCl was neutralized with 500 μl of 1 N NaOH. The production of L-aspartic acid was quantitated as described in Methods. A single circlet of Dacron bearing ~0·1 i.u. L-asparaginase was used for determining the Michaelis constant of the bound enzyme at each of the pH values listed. Incubations were carried out at 37° in 2 ml substrate agitated at the rate of 100 back-and-forth strokes/min. Incubation times ranged from 2 to 20 min but in no case was more than 20 per cent of the substrate hydrolyzed. The production of L-aspartic acid was measured spectrophotometrically, as described above.

charged enzyme and substrate. But, L-asparagine bears only a feeble negative charge at a pH of 8·4. It is, therefore, unlikely to be attracted to the stationary enzyme by net electrostatic forces. Charge effects, then, probably do not explain the altered K_m of L-asparagine which results from covalent conjugation. Similarly, the neutrality of L-asparagine probably explains our inability to observe any influence of ionic strength on the Michaelis constant.

Susceptibility to product inhibition. Ammonium ions and L-aspartic acid both are known to inhibit free L-asparaginase from *E. coli*: the former product is a relatively powerful inhibitor, the latter a relatively feeble inhibitor. When the product inhibition of L-asparaginase coupled to a Dacron support was examined, similar effects were seen. Ammonium ions were found to inhibit both the free and bound enzymes in a non-competitive way, whereas L-aspartic acid inhibited both enzymes competitively (Table 4). Moreover, the quantitative aspects of the inhibition by both reaction products were found to be quite similar in the case of the free and bound species.

Thermostability of free L-asparaginase and of L-asparaginase bound to Dacron. In view, too, of the reports of the resistance of L-asparaginase from *E. coli* to thermal denaturation [16], it was felt to be of interest to explore the stability of the Dacron-bound enzymes in solutions exposed to temperatures ranging from 27 to 97°. For this purpose, small circlets were cut from the tubular pieces of Dacron, and their content of L-asparaginase was determined by repeated assays. The circlets of bound enzyme then were exposed once to the temperatures listed in Fig. 3 (left panel) and reassayed at 37°. It can be seen that a small but significant percentage of the enzymatic acitivity of the bound but not of the free enzyme is lost by exposure to a temperature of 50°. Higher temperatures fractionally destroy both enzymes, in an apparently irreversible way. When the

Table 4. Product inhibition of free and bound L-asparaginase*

Product	Free enzyme		Bound enzyme	
	K_i (M)	Apparent type of inhibition	K_i (M)	Apparent type of inhibition
L-Aspartic acid	0·14	Competitive	0·124	Competitive
NH_4Cl	0·008	Non-competitive	0·013	Non-competitive

* Inhibition of free and bound L-asparaginase by NH_4Cl was studied at concns of product between 0·005 and 0·01 M; inhibition by L-aspartic acid was studied at concns of product between 0·1 and 0·2 M. When NH_4Cl was the inhibitor, the hydrolysis of L-asparagine to L-aspartic acid was quantitated by an enzymatic spectrophotometric technique [6]. Ammonium ions do not interfere with this technique. However, when L-aspartic acid was being examined as an inhibitor, it was not possible to obtain reliable results with the enzymatic technique customarily used to measure ammonia. Therefore, a radiometric assay was used: in a final volume of 1 ml were admixed the appropriate concentrations of L-asparagine (with or without L-aspartic acid) and 100,000 cpm of UL-[^{14}C]L-asparagine, sp. act. 156 μCi/μmole. L-Asparaginase, free or bound, was added in a concn and for a time sufficient to hydrolyze no more than 20 per cent of the substrate. The reactions were terminated by the addition of one-tenth volume of 1 N HCl or removal of the graft, and the liberated UL-[^{14}C]L-aspartic acid was quantitated in a small aliquot by the technique given in Ref. 10, except that the analytical mixture contained a 10-fold increase in the concn of α-ketoglutarate to accommodate the proportionately increased concn of L-aspartic acid present in these reaction mixtures.

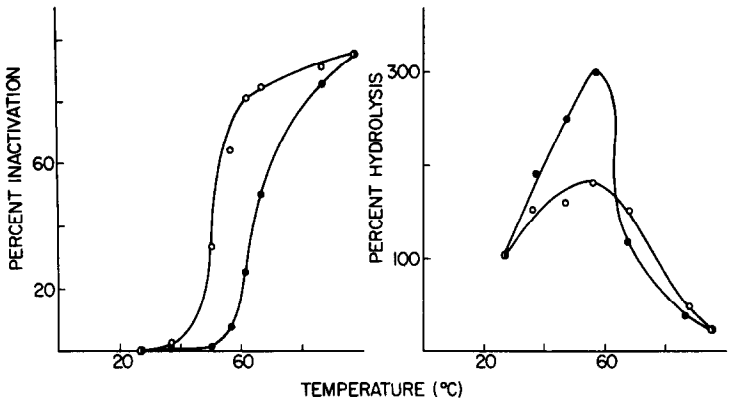

Fig. 3. Response of free and bound L-asparaginase to increments of temperature. *Left panel*: For studies of the inactivation of free L-asparaginase (●), solutions of L-asparaginase, 20 i.u./ml of 0·50 M Tris-HCl, pH 8·45, were incubated at the temps indicated for 10 min, and thereafter an appropriate aliquot was taken for the measurement of L-asparaginase activity at 37°, as described in Methods.

Right panel: For studies of the hydrolytic activity of free L-asparaginase over the range of temps indicated, solutions of 0·01 M L-asparagine in 0·05 M Tris-HCl at pH 8·45 were prewarmed to the requisite temp, whereafter a suitable aliquot of L-asparaginase (usually 0·1 i.u.) was added and hydrolysis allowed to proceed for 10 min. One-tenth volume of 1 N HCl was used to terminate the reaction; after its addition, the reaction vessels were immediately iced to minimize "blank" hydrolysis of L-asparagine. Ten min later, the HCl was neutralized with 1 N NaOH. The measurement of L-aspartic acid is described in Methods. Results are expressed as per cent of the activity measured at 27°.

Studies on the inactivation and hydrolytic velocity of the bound enzyme (○) were carried out in an analogous manner, except that the reactions were terminated by removing the circlets bearing the L-asparaginase, and icing the reaction vessels.

hydrolytic velocity of bound L-asparaginase was contrasted with that of the free enzyme over a wide range of temperatures including denaturing ones, it was found that both species were apparently better catalysts at 57° than at 37° (Fig. 3, right panel). Since it has already been demonstrated that some inactivation of the bound enzyme is occurring at this temperature, it must be concluded that the surviving enzyme molecules are catalyzing the hydrolysis of L-asparagine at rates even greater than those tabulated. It is noteworthy that serum albumin failed to shield the bound enzyme from inactivation by heat and also produced no enhancement of its activity. On the other hand, albumin offers the free enzyme significant protection against thermal destruction, and stimulates it significantly.

When the influence of temperature on the affinity of an enzyme-bearing graft for L-asparagine was studied, K_m values of 3×10^{-3} M, 6×10^{-3} M, 6×10^{-3} M and 4×10^{-3} M were measured at 0, 25, 37 and 50° respectively. Thus, this span of temperature produced no major alteration of the Michaelis constants.

Interaction with L-DONV. Free L-asparaginase from *E. coli* can decompose L-DONV to N_2 and 5-hydroxy-4-oxo-L-norvaline; in addition, the free enzyme can be alkylated by L-DONV, presumably at its catalytic site, and the alkylated L-asparaginase is nearly inactive [7]. Conjugation does not essentially alter these properties: bound L-asparaginase actively decomposes this diazoketone and is, in addition, highly susceptible to inactivation by L-DONV. Thus, immersion of a circlet of Dacron to which L-asparaginase is covalently bonded in 0·0024 M L-DONV brings about a progressive decline in amidohydrolytic activity of the graft (Fig. 4). Exposure to L-DONV in 50% DMSO accelerates the rate of inactivation. It is also noteworthy that the decline in activity falls short of total inactivation in both milieus, a situation analogous to that seen when the free enzyme is exposed to this diazoketone. DMSO was used because Jackson and Handschumacher [7] have reported that the rate of catalytic decomposition of L-DONV is reduced to a minimum in 50% buffered dimethylsulfoxide, whereas the rate of alkylation of the enzyme is increased to a maximum. In order to compare the bound with the free enzyme, therefore, incubations of bound L-asparaginase with [^{14}C]L-DONV were carried out in 50% DMSO, and the degree of alkylation was assessed by scintillation spectrometry. In contradistinction to the case with free L-asparaginase, buffered solutions of DMSO failed to enhance alkylation of the bound species by L-DONV. Thus, the number of moles of DONV bound/mole of L-asparaginase on these grafts was 1·08 irrespective of solvent. Since the free enzyme is a tetramer and has been shown to bind 1 mole of L-DONV/monomeric subunit, the present result raises the possibility that the process of conjugation dissociates L-asparaginase into monomers. Alternatively, because a large fraction of

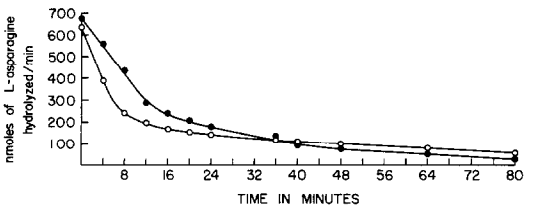

Fig. 4. Time–course of the inactivation by L-DONV of L-asparaginase covalently conjugated to a Dacron prosthesis: the influence of DMSO. After measurement of its native content of L-asparaginase, a circlet of Dacron bearing 0·67 i.u. was incubated in 0·0024 M L-DONV in 0·1 M potassium phosphate, pH 7·4 (●). A second circlet of Dacron bearing 0·63 i.u. L-asparaginase was incubated in 0·0024 M L-DONV in a binary mixture of buffer and DMSO (50:50, v/v) (○). Incubations were for 4 min, after which the circlets were rinsed, incubated in 0·01 M L-asparagine in 0·05 M Tris-HCl, pH 8·4, for 2 min, rinsed and reincubated in the appropriate solution of DONV for 4 min, reassayed vs L-asparagine, etc., until a total time of exposure to L-DONV of 80 min had elapsed. The generation of L-aspartic acid was quantitated spectrophotometrically [6]. In the course of the incubation, 50 per cent of the L-DONV was catalytically decomposed by the graft so that the final molarity of inhibitor was 0·0012 M, and the mean molarity prevailing during the alkylation was 0·0018 M.

the protein coupled to Dacron prostheses such as these was found to be enzymatically inert, the denatured molecules may have been responsible for the deviation observed.

In the case of conjugated L-asparaginase, considerable hydrolytic activity remained after treatment with L-DONV. It was felt to be important to characterize

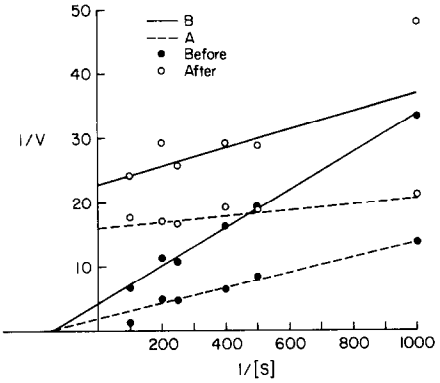

Fig. 5. Apparent Michaelis constants of *E. coli* L-asparaginase covalently bound to Dacron, before and after alkylation with L-DONV. After duplicate measurements of their K_m, grafts A and B were alkylated by exposure to 5×10^{-3} M L-DONV in 0·1 M potassium phosphate, pH 7·4, for 1 hr at 37°. Thereafter, the affinity constants were again measured, as described in the legend of Table 1. The ordinate indicates the reciprocal of the velocity, μmoles L-asparagine hydrolyzed/min graft.

the kinetic aspects of this residual activity in order to determine if surviving enzyme was intact or had, in fact, been subtly altered by exposure to the alkylating substrate. As is shown in Fig. 5, L-DONV-treated L-asparaginase exhibited a markedly depressed V_{max} toward L-asparagine, but its affinity for this substrate was remarkedly improved: the K_m had decreased by a factor of 10. This improvement in affinity was seen with four separate preparations of conjugated L-asparaginase, although some small variability in the degree thereof was observed (Table 5). Interestingly, the K_m of the bound alkylated enzyme for β-cyano-L-alanine was virtually unchanged. By contrast, free L-DONV-inactivated enzyme was found to exhibit a K_m for L-asparagine >1 log higher than its unalkylated counterpart.

Response of bound L-asparaginase to a variety of modifiers. When free L-asparaginase is incubated with Pronase, amidohydrolytic activity is quickly lost. Citri *et al.* [17, 19] have made a comparable observation; in addition, they have reported that L-asparagine can partially protect against inactivation. In our studies of the effect of proteolysis, we have observed that three amino acids and at least several peptides are released from the free enzyme within 5 min of exposure to Pronase at a concentration of 50 μg/ml thus, 0·218 mole L-lysine, 2·84 moles L-tyrosine and 3·7 moles L-aspartic acid appear/mole of L-asparaginase. Electrophoresis of the treated enzyme on slabs of acrylamide gel in Tris-borate buffer at pH 8 reveals that even 2 min of proteolytic attack is sufficient to alter the mobility of the enzyme. This finding indicates that the mass of free L-asparaginase is reduced substantially, its net negative change increased, or both, by digestion with Pronase. To investigate further the influence of Pronase on Dacron-bound L-asparaginase, the rate of inactivation and the amino acid composition of the incubation medium were determined sequentially during proteolysis (Fig. 6). It can be seen that the rate of inactivation of bound L-asparaginase is significantly slower than that of the free enzyme. As was the case with free L-asparaginase, the amino acid released in the greatest abundance during digestion of the bound species by 50 μg Pronase was L-aspartic acid; in both cases, too, L-tyrosine was the next most abundant product. But, whereas early proteolysis of the free enzyme liberated only three amino acids, similar conditions of digestion of the enzyme coupled to Dacron resulted in the release of all of the constituent amino acids of the protein except L-proline and L-cysteine. The greater susceptibility of conjugated L-asparaginase to digestion is at variance with its comparative resistance to enzymic inactivation by Pronase. This discrepancy may reflect the significant fraction of inert or denatured protein present in the preparations used, a fraction presumably more prone to proteolysis than the catalytically intact species.

Of the other putative modifiers which were examined in the present experiments, tyrosinase at a concentration of 100–1000 μg/ml, in the presence or absence of elemental iodine, failed to inhibit or destroy

Table 5. Changes in the Michaelis constants of free and conjugated L-asparaginase toward prototypical substrates after alkylation with L-DONV*

Substrate	K_m (M)			
	Conjugated L-asparaginase		Free L-asparaginase	
	Before alkylation	After alkylation	Before alkylation	After alkylation
L-Asparagine	$3{\cdot}3 \times 10^{-3} \pm 2{\cdot}2 \times 10^{-3}$†	$4{\cdot}5 \times 10^{-4} \pm 2{\cdot}6 \times 10^{-4}$†	$3{\cdot}0 \times 10^{-5}$	$3{\cdot}3 \times 10^{-4}$‡
β-Cyano-L-alanine	$1{\cdot}2 \times 10^{-2} \pm 7{\cdot}0 \times 10^{-3}$§	$9{\cdot}8 \times 10^{-3} \pm 5{\cdot}54 \times 10^{-3}$§	$7{\cdot}8 \times 10^{-3} \pm 3{\cdot}3 \times 10^{-3}$‖	$2{\cdot}4 \times 10^{-2} \pm 1{\cdot}3 \times 10^{-2}$‖
L-DONV	$5{\cdot}0 \times 10^{-4}$¶	$5{\cdot}0 \times 10^{-4}$¶	$1{\cdot}0 \times 10^{-5}$‡	$8{\cdot}0 \times 10^{-5}$‡

* Apparent Michaelis constants for L-asparagine, β-cyano-L-alanine and L-DONV were determined in 0·05 M Tris-HCl, pH 8·4, over an appropriate range of concns as described in the legend to Table 1. Free L-asparaginase was the enzyme from *E. coli* used as such or alkylated with L-DONV in 0·1 M potassium phosphate DMSO (1:1, v/v), as described in Ref. 7. This enzyme was ~99 per cent inactivated.

† Mean of eight determinations with five preparations.

‡ One determination with the preparation of Handschumacher (cf. Materials).

§ Mean of three determinations with three preparations.

‖ Mean of two determinations with the preparation of Handschumacher (cf. Materials).

¶ Mean of two determinations with two preparations.

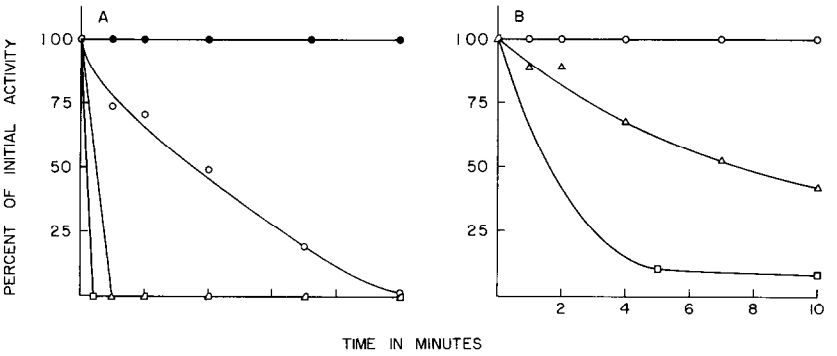

Fig. 6. Influence of Pronase on the activity of free and bound L-asparaginase. Samples of free (A) and bound (B) L-asparaginase (10 i.u.) were incubated for the times indicated with solutions of Pronase prepared in 0·005 M Tris-HCl, pH 8·4, rendered 0·001 M in $CaCl_2$. The Pronase concns were 50 μg/ml (□——□), 5 μg/ml (△——△), 0·5 μg/ml (○——○) and 0·05 μg/ml (●——●). After incubation with Pronase, one aliquot of the solution of free L-asparaginase was diluted 1000-fold in 0·01 M L-asparagine, made up in 0·05 M Tris-HCl, pH 8·4, to terminate proteolysis and initiate the assay of residual L-asparaginase activity. Another aliquot was acidified with one-tenth volume of 1 N HCl. This sample was used for amino acid analysis on the long column of the Jeol amino acid analyzer. After incubation with Pronase, the bound enzyme was washed with several large volumes of deionized water. The residual activity of L-asparaginase was determined by incubating the bound enzyme with 2 ml of 0·01 M L-asparagine made up in 0·05 M Tris-HCl, pH 8·4. The incubation was carried out in an oscillating water bath maintained at 37° for 2 min. The hydrolytic activity of both the free and bound L-asparaginase was measured by L-aspartic acid production as described in Methods.

free or covalently coupled L-asparaginase during a 30-min incubation at 37° in 0·05 M Tris-HCl at pH 6·4. This negative result is possibly due to the inability of tyrosinase to attack the L-tyrosyl residues of L-asparaginase, although the former enzyme is able to oxidize L-tyrosyl residues of many other proteins. It is noteworthy that several groups have, in fact, demonstrated that L-tyrosine is essential to the function of L-asparaginase from *E. coli* [20–23].

On the other hand, after 30 min of exposure to 6 M urea at neutral pH and 37°, the coupled L-asparaginase was significantly and irreversibly inactivated. Thus, permanent changes in the tertiary structure of the enzyme could still be accomplished despite the constraint imposed by chemical conjugation.

Detachment of covalently bound L-asparaginase from the Dacron prosthesis. Under ideal circumstances insolubilized enzymes ought to be chemically conjugated to their support in such a way that hydrolysis, or detachment by another reaction, is precluded. In our initial studies with the Dacron-to-L-asparaginase bond, we were unable to detect leakage of the enzyme during storage. Indeed, the grafts retained over 87 per cent of their starting activity after 3 months in physiological saline at 4° and 51 per cent after 27 months. These findings were felt to substantiate the stability of the linkage in question. With the advent of more sensitive techniques of measuring L-asparaginase, however, the question of detachment of enzyme was reinvestigated. A radiometric assay capable of detecting 1×10^{-6} i.u. of the enzyme was utilized. It was found that at 37°, in the presence of 0·01 M L-asparagine, L-glutamine or β-cyano-L-alanine, 5×10^{-5} i.u. (~0·01 per cent) of enzyme was detached during a 20-min incubation of a 1-cm graft. In the presence of 0·001 M L-DONV, the alkylating substrate, enzyme was also detected in the perfusate, but quantitation of it was rendered difficult by the inhibitory effect of L-DONV on hydrolytic activity. It was also observed, at temperatures below denaturing ones, that the rate of detachment was temperature dependent, insofar as this could be assessed.

Immunologic studies. Antibodies to free L-asparaginase from *E. coli* are ordinarily able to diminish its activity (V_{max}) by ~50 per cent; but total inactivation does not result even from long exposure of the enzyme *in vitro* to antisera of high titre [1]. *In vivo*, however, the presence of antibodies to L-asparaginase is accompanied by prompt removal of the enzyme from the blood stream and the oncolytic action of the enzyme is concomitantly nullified [24]. Since it was possible that covalent conjugation of L-asparaginase to Dacron had occluded certain of its antigenic sites, we felt that it would be of interest to determine the influence of mouse anti-L-asparaginase antibodies on the V_{max} of the bound enzyme. Pooled mouse serum with a 1:512 titre, as assessed by passive hemagglutination, was used for this study. Only undiluted serum inactivated the bound enzyme and the degree of inactivation ranged from 30 to 52 per cent in three separate experiments with the same serum pool. While the reason for the difference in titre between the two different assays is unclear, it can be concluded that antibodies to the enzyme can compromise the activity of the graft.

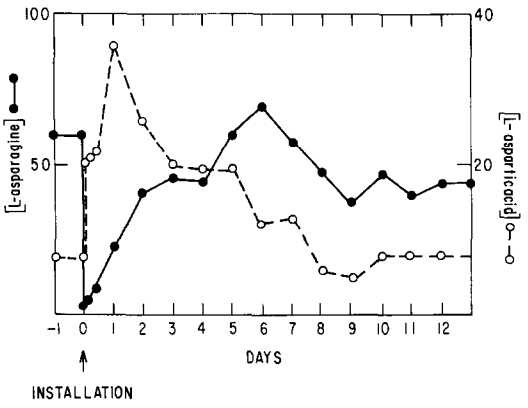

Fig. 7. Influence of a Dacron vascular prosthesis bearing covalently coupled L-asparaginase and inserted in a great vein on the concentration of L-asparagine and L-aspartic acid in the dog. Measurements of L-asparagine (●—●) and L-aspartic acid (○—○) were carried out spectrophotometrically as described in Methods. On day 0 (vertical arrow) a prosthesis bearing 10·3 i.u. L-asparaginase from *E. coli* was installed in the inferior vena cava, as described in Methods. Five-ml samples of heparinized blood were taken at the times indicated, and plasma was separated from them at once in the cold. The plasma was either boiled immediately in well-capped polycarbonate tubes, or frozen at once and boiled at the time of assay. Units are in nmoles/ml. Not shown in the figure, but appended here in the interest of completeness, is the influence of the graft on the plasmatic ammonia and L-glutamine of the recipient.

Day	Time of sample condition	Hr (a.m.)	NH_3	L-Glutamine (nmoles/ml)
−1	Pre-op	8	86	817
0	Pre-op	8	191	695
0	Post-op	10	121	469
0	Post-op	12 noon	139	521
0	Post-op	2	104	486
1	Post-op	8	139	765
3	Post-op	8	171	765
4	Post-op	8	208	886
5	Post-op	8	173	765
6	Post-op	8	156	643
8	Post-op	8	139	939
9	Post-op	8	156	782
10	Post-op	8	156	939
11	Post-op	8	121	852
12	Post-op	8	104	1008

Pursuant to these findings, attempts were made to detect antibodies to L-asparaginase *in vivo*. As adjudged by the response of their passive hemagglutination titres after intraperitoneal installation of grafts bearing 1 i.u. of enzyme, experimental mice were in fact immunized to the enzyme under the circumstances of this study. Thus the passive hemagglutination titre of graft recipients was zero on the 1st day after implantation, rose to detectable levels on day 12 (1:4–1:32), increased further by the time 32 days had elapsed (1:16–1:32), and was maintained at that level for at least another 14 days. Thus, it can be presumed that leakage of L-asparaginase occurred *in vivo* by analogy to the studies *in vitro* described earlier.

Additional studies in vivo. Non-heparinized, enzyme-bearing grafts installed in a common iliac artery of dogs remained patent for at least 10 days, although with progressive diminution of the ipsilateral pulses. These arterial prostheses failed to lower the level of plasmatic L-asparagine. When removed, 3 weeks after placement, these arterial grafts were enzymatically inactive, and covered with fibrinous deposits. Conversely, installation of a graft bearing covalently bound L-asparaginase and heparin in the upper inferior vena cava did produce significant but transitory depressions of the concentration of L-asparagine in plasma (Fig. 7) and did perturb the manner in which exogenous L-asparagine was handled by the recipient. For these studies, 4-[^{14}C]L-asparagine was injected into the experimental subject, before and after the installation of the Dacron prosthesis. It was reasoned that an enzymatically active graft would catalyze the degradation of the radioactive L-asparagine to 4-[^{14}C]L-aspartic acid which can be discriminated from L-asparagine in the radiometric assay system used [10]. The results of this study are illustrated in Fig. 8. L-Asparagine was the sole radioactive species detected in the clearance studies carried out before surgery; L-aspartic acid was virtually the sole species detected 6 hr after surgery. At this time period, too, the net concentration of [^{12}C]L-asparagine in plasma was markedly reduced and that of [^{12}C]L-aspartic acid correspondingly elevated. The most significant feature of Fig. 8, however, is the observation that 4-[^{14}C]L-aspartic acid was still being generated from the injectate at a significant rate even 10 days after engraftment. This finding is taken as evidence for the enzymatic integrity of the graft. That some L-asparaginase has been lost or inactivated, however, is shown by the failure of the graft to eradicate 4-[^{14}C]L-asparagine or [^{12}C]L-asparagine totally at this later time period, in contradistinction to its performance immediately after implantation.

Therapeutic studies in mice. In order to test the therapeutic activity of the covalently conjugated enzyme, circlets of Dacron bearing 1 i.u. of enzyme were implanted in the peritoneal cavity of five BDF_1 mice, while five controls received the graft minus enzyme. Six hr later, both groups received intraperitoneal injections of 1×10^5 cells of leukemia 5178Y sensitive to L-asparaginase. No difference in survival times between these two experimental groups was observed (the median survival time was 14 days for both groups). All subjects developed massive ascites. At autopsy, the circlets were tenaciously adherent to the serosa of the small and large intestines. After removal of the fibrous adhesions, 15 per cent of the original enzymic activity was still present. Sub-passage of ascites cells from subjects bearing the enzyme graft, and challenge of the recipients with L-asparaginase revealed that the tumor cells which had grown in the presence of the prosthesis were still sensitive to the

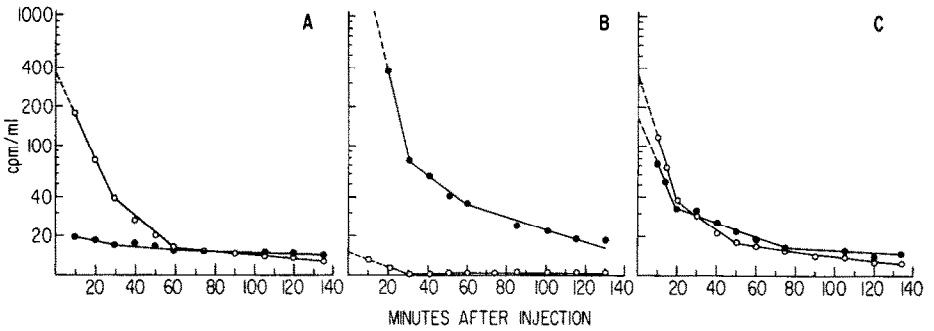

Fig. 8. Clearance of 4-[^{14}C]L-asparagine from the plasma of a dog bearing a Dacron prosthesis to which L-asparaginase was covalently coupled. A total of three clearance studies were carried out, essentially as described in Ref. 10. Clearance A was performed on the day before surgery; clearance B 6 hr after the surgical implantation of the prosthesis as described in Methods; and clearance C on day 10 after implantation. For the conduct of the clearance studies, 20 μCi 4-[^{14}C]L-asparagine was injected intravenously at zero time, and blood samples were taken from a contralateral vein at the times indicated. Plasma was separated at once in the cold and frozen. The measurements of 4-[^{14}C]L-asparagine and 4-[^{14}C]L-aspartic acid were carried out radiometrically [10]. Key: (●) represents 4-[^{14}C]L-asparagine; (○) represents 4-[^{14}C]L-aspartic acid.

oncolytic action of L-asparaginase. It appeared likely, on this basis, that the adhesions had abrogated the ability of the grafts to depress the concentration of L-asparagine in the ascitic fluid. Direct measurements of L-asparagine in the ascitic fluid revealed that this was the case: in the controls the mean concentration of ascitic L-asparagine was 65 nmoles/ml and in the recipients of the L-asparaginase graft the corresponding value was 61 nmoles/ml.

DISCUSSION

Despite the fact that L-asparaginase exhibits a very high order of oncolytic activity against acute lymphocytic leukemia in children, its toxicity has proven to be a drawback to its routine use. One of the most significant and frequent toxic actions of the enzyme has been its production of anaphylaxis, occasionally with fatal outcome. It would not be surprising, of course, for this large and alien protein to be antigenic, except for the repeated observations that L-asparaginase is, in its own right, a powerful immunosuppressant. This latter feature, in fact, may have helped to minimize the number of anaphylactic reactions seen during therapy with the enzyme. The antigenicity of L-asparaginase also provides for a very powerful mechanism of resistance to its oncolytic action: antibodies sharply diminish the hydrolytic potency of L-asparaginase, and bring about a dramatically accelerated clearance of the enzyme from the bloodstream of recipients. The rate of clearance of L-asparaginase influences its therapeutic efficiency: slow clearance promotes oncolysis. Thus, this immunologically accelerated clearance is an undesirable occurrence.

With these facts in mind, attempts have been made in the present study to examine the feasibility of bonding L-asparaginase to an inert support in the hopes of circumventing its antigenicity. Dacron vascular prostheses were natural candidates for this approach in view of their demonstrated utility in numerous clinical trials.

When arterial placements of grafts bearing only L-asparaginase were attempted, plasma protein accumulated on the intimal face of the graft, thus interfering with amidohydrolysis. Studies *in vitro* with similar grafts through which heparinized blood was pumped illustrated how rapidly a protein coat could build up and impede amidohydrolysis. Indeed, although these non-heparinized grafts were inert at the time of excision, 20 days after surgery, it is possible that L-asparagine, the substrate for the bonded enzyme, simply could not diffuse through the accumulation of protein for its hydrolysis to be detectable under the circumstances of the assay.

When heparin and L-asparaginase were coupled to the grafts, they appeared to deplete circulating L-asparagine, at least transitorily. It is likely that the venous placement enhanced this effect, inasmuch as the blood of the inferior vena cava is enriched with the effluents from many organs; thus the enzyme would be more saturated with its substrate under these circumstances. Moreover, when radiolabeled L-asparagine was injected into the graft recipient, in order to probe the dynamic features of the disposition of the amide, its removal was markedly perturbed, both on the day of installation of the graft and 10 days later. At this time, the rate of removal of L-asparagine was only slightly accelerated, but L-aspartic acid accumulated, a finding indicative of on-going amidohydrolysis. Despite this evidence of graft function, the plasmatic concentration of L-asparagine was being maintained within normal limits at this latter time, so that compensating output

by the L-asparagine-synthesizing sites in the organism must be postulated [25].

The biochemical changes produced by bonding L-asparaginase to Dacron reflect the physical chemistry of the conjugated species: the enzyme has been fixed in a domain that is both impermeable and hydropenic (at least on one side). The unilateral impermeability explains the dependence of V_{max} on flow; unilateral hydrophobicity explains the modified response of the bonded enzyme to exogenous hydropenic environments; that is to say, being already confined in a hydropenic environment, the enzyme cannot respond prototypically to further hydropenia by analogy to other insolubilized enzymes [14]. The local alkalinity of the diazotized Dacron explains the shift in the pH optimum vs L-asparagine from alkaline to neutral. Since L-threonine or L-serine is very likely involved both in the conjugation of the enzyme to Dacron and in the act of catalysis [26], it is reasonable to speculate that the marked elevation of K_m values seen after conjugation is a result of elimination of a strategic hydroxyl function. Evidence that this elimination has made the active site more rather than less spacious is provided by the finding of an augmented L-glutaminase activity of the conjugated species.

While these studies were in progress, several publications appeared documenting the biochemical properties of L-asparaginase coupled to various inert supports [27-30]. The results of these studies are essentially in accord with the results of the present study, with one notable exception, however; Horvath *et al.* [30] have found that their preparation of immobilized L-asparaginase exhibits a K_m for L-asparagine identical to that of the free enzyme. As has been demonstrated here, the affinity of the Dacron-bound enzyme for its principal substrate is depressed to a significant degree. The cause of these differences in affinity may arise from the different techniques used to immobilize the enzyme.

In summary, although the L-asparaginase graft has yet to undergo therapeutic trials, it did exhibit several biochemically important attributes: relatively stable amidohydrolytic activity *in vivo* and the ability to deplete the plasma of L-asparagine for brief periods. If greater unitage of the enzyme and of heparin or even of a fibrinolysin can be coupled to Dacron, there is reason to anticipate that an oncolytic prosthesis will result. However, the problem of antigenicity remains to be solved. Toward this end, a more durable conjugate is being sought.

Acknowledgements—The authors would like to thank Dr. R. Peterson for performing the passive hemagglutination titres. Dr. Ronald Zlotoff helped in the execution and tabulation of many of the experiments presented here. Miss M. Gaston is thanked for her help in the immunologic studies, and Mr. Chip Manning for his help with the rheologic studies. The surgical assistance of Dr. Alfred Cohn is gratefully acknowledged, as is the excellent technical help of Mr. Harry Milman. Mrs. Ruth Davis was of material assistance at every stage in the preparation of the manuscript; lastly, Mrs. Linda Klipp and Mrs. Audrey Loveridge are thanked for their careful typing of the text.

REFERENCES

1. R. G. Peterson, R. E. Handschumacher and M. S. Mitchell, *J. clin. Invest.* **50,** 1080 (1971).
2. R. L. Capizzi, J. R. Bertino, R. T. Skeel, W. A. Creasey, R. Zanes, C. Olayon, R. G. Peterson and R. E. Handschumacher, *Ann. intern. Med.* **74,** 893 (1971).
3. A. Khan and J. M. Hill, *J. Lab. clin. Med.* **73,** 846 (1969).
4. H. H. Weetall, *J. Biomed. Mater. Res.* **4,** 597 (1970).
5. L. S. Hersh and H. H. Weetall, *J. Am. chem. Soc.* **3,** 85 (1970).
6. D. A. Cooney, R. L. Capizzi and R. E. Handschumacher, *Cancer Res.* **30,** 929 (1970).
7. R. C. Jackson and R. E. Handschumacher, *Biochemistry* **9,** 3585 (1970).
8. D. A. Cooney, R. D. Davis and G. VanAtta, *Analyt. Biochem.* **40,** 312 (1971).
9. H. N. Jayaram, T. Ramakrishnan and C. S. Vaidyanthan, *Indian J. Biochem.* **6,** 106 (1969).
10. D. A. Cooney, E. R. Homan, T. Cameron and U. Schaeppi, *J. Lab. clin. Med.* **81,** 455 (1973).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
12. J. S. Holcenberg, *Biochim. biophys. Acta* **185,** 228 (1969).
13. N. Nakamura, Y. Morikawa and M. Tanaka, *Biol. Chem.* **35,** 743 (1971).
14. G. J. H. Melrose, *Rev. pure appl. Chem.* **21,** 83 (1971).
15. J. C. Wriston and T. Yellin, *Adv. Enzymol.* **39,** 185 (1973).
16. D. A. Cooney and R. D. Davis, *Biochim. biophys. Acta* **212,** 134 (1970).
17. N. Citri and N. Zyk, *Biochemistry* **11,** 2103 (1972).
18. O. Zaborsky, in *Immobilized Enzymes* (Eds. R. C. Weast, S. M. Selby, J. W. Long and I. Sunshine), p. 175. CRC Press, Ohio (1973).
19. N. Citri, N. Kitron and N. Zyk, *Biochemistry* **11,** 2110 (1972).
20. S. Shifrin and B. G. Solis, *J. biol. Chem.* **247,** 4121 (1972).
21. Y. Nishimura, H. Makino, O. Takenaka and Y. Inada, *Biochim. biophys. Acta* **227,** 171 (1971).
22. L. P. Liu and R. E. Handschumacher, *J. biol. Chem.* **247,** 66 (1972).
23. J. Makino, O. Takenaka and Y. Inada, *Biochim. biophys. Acta* **263,** 477 (1972).
24. A. I. Goldberg, D. A. Cooney, J. P. Glynn, E. R. Homan, M. R. Gaston and H. A. Milman, *Cancer Res.* **33,** 256 (1973).
25. J. S. Woods and R. E. Handschumacher, *Am. J. Physiol.* **4,** 224 (1973).
26. J. G. Gumprecht and J. C. Wriston, *Biochemistry* **12,** 4869 (1973).
27. J. P. Allison, L. Davidson, A. Gutierrez-Hartman and G. B. Kitto, *Biochem. biophys. Res. Commun.* **47,** 66 (1972).
28. T. M. S. Chang, *Nature, Lond.* **229,** 117 (1971).
29. F. X. Hasselberger, H. D. Brown, S. K. Chattopadhyay, A. N. Mather, R. O. Stasiw, A. B. Patel and S. N. Pennington, *J. Cancer Res.* **30,** 2736 (1970).
30. C. Horvath, A. Sardi and J. S. Woods, *J. appl. Physiol.* **34,** 181 (1973).