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THE EFFECT OF CHEMICAL MODIFICATION OF L-ASPARAGINASE ON ITS PERSISTENCE IN CIRCULATING BLOOD OF ANIMALS

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Abstract—The persistence in circulating blood of rabbits and mice of *Erwinia carovotora* asparaginase and some of its chemically modified derivatives was compared. Derivatives of various molecular size were obtained by reacting the enzyme with some bifunctional reagents and activated dextrans. The persistence of the larger molecular weight derivatives of the enzyme was many-fold higher than that of the native enzyme. Radioactive labelling of the enzyme showed that it may be removed intact from the circulation.

Streptokinase from a Streptococcus is widely used as a thrombolytic agent and asparaginase from *Escherichia coli* and *Erwinia carotovora* are being used for the treatment of acute lymphatic leukaemia [1]. The wider intracorporeal use of microbial enzymes is impeded by their rapid clearance from the circulating blood, a feature they share with other heterologous proteins. The mechanism of clearance has not been elucidated but it is known to be influenced by the species of animal host and the physico-chemical properties of the protein [2]. The relative importance of these properties is not clear although electrostatic charges [3, 4] and molecular size are among those [5] believed to influence it. The most significant factor affecting asparaginase clearance in mice was shown by Riley *et al.* to involve the presence of lactic dehydrogenase virus [6]; asparaginase injected into mice previously inoculated with this virus disappeared from the blood at a significantly slower rate than in corresponding virus-free mice. This phenomenon is not understood.

In an attempt to determine the importance of molecular size in this process polymers of asparaginase from *E. carotovora* were prepared using bifunctional and polyfunctional reagents. The products of these reactions were fractionated and the various polymers examined to identify those properties that influence persistence.

E. carotovora asparaginase is an oligomeric protein composed of four identical subunits (protomers). Various derivatives of the enzyme were produced by reacting with a bifunctional compound: dimethylsuberimidate, glutaraldehyde, *N*-ethyl-5-phenylisoxazolium-3′-sulphonate (Woodward reagent K) or with periodate activated dextrans of various molecular weights. Dimethylsuberimidate and glutaraldehyde and periodate activated dextrans react with lysine groups of asparaginase producing three types of covalent cross linkages: (1) intramolecular bonds within each protomer, (2) intramolecular bonds formed between different protomers of the same molecule and (3) intermolecular bonds between protomers of different molecules forming multiple molecular weight polymers of asparaginase. It may also be possible for each dimethylsuberimidate molecule to react with only one lysine group, the remaining imidoester group being hydrolysed to an ester group instead of reacting with another lysine group forming a cross linkage.

Intra- and intermolecular peptide bonds were formed when asparaginase was reacted with the Woodward reagent. In this case the covalent cross linkages were formed by condensation of existing carboxylic and primary amino side chains [7]. In contrast, the covalent linkages created by dimethylsuberimidate, glutaraldehyde and activated dextrans originated from the reagent molecules.

MATERIALS AND METHODS

L-Asparaginase. Freeze-dried *E. carotovora* asparaginase was obtained in ampoules (MRE Porton, Salisbury, Wilts) each containing about 10 mg protein (specific activity 550 units/mg protein; a unit releases 1 μmol ammonia per min at 37° at pH 8.5). The enzyme was dissolved in the appropriate buffer solution immediately before use.

Chemical modification of asparaginase

Reaction with dimethylsuberimidate. Dimethylsuberimidate was prepared from suberonitrile (Koch-Light Laboratories, Colnbrook, Bucks) by the method of McElvain and Schroeder [8]. Preliminary tests were conducted to establish the optimum conditions required to produce active and soluble modified asparaginase using methods described by Davies and Stark [9] and Handschumacher and Gaumond [10].

The maximum conversion of enzyme commensurate with the retention of solubility was obtained by adding 120 mg dimethylsuberimidate to 720 mg asparaginase in 120 ml of 0.2 M triethanolamine hydrochloride buffer, pH 8.5, at 0°. The pH was maintained by adding 0.01 M NaOH. After 4 hr the reaction was stopped by adding 1.2 ml glacial acetic acid dropwise to the stirred suspension to bring the pH down to 4.5. Excess reagents and by-products of the reaction were removed by dialysis against three 5-litre volumes of 0.1 M sodium phosphate buffer, pH 7.4. The product was concentrated to about 5 ml by pressure dialysis using an Amicon Model 52 ultrafiltration cell incorporating a

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PM Diaflow membrane (Amicon Ltd., High Wycombe, Bucks).

Reaction with glutaraldehyde. Low concentrations of glutaraldehyde were used to avoid precipitation of the enzyme. Fully active polymers were produced by reacting 20 mg protein and 42μg glutaraldehyde per ml 0.1 M sodium phosphate buffer, pH 7.4. The glutaraldehyde (100μl of 25% w/v solution obtainable from Merk, Sharpe & Dohme Ltd., Hoddesden, Herts.) was added to the stirred solution of asparaginase (60 ml) and the mixture incubated for 4 hr at room temperature. The product was dialysed and concentrated as described above.

Reaction with N-ethyl-5-phenylisoxazolium-3'-sulphonate (Woodward reagent K). Rapid precipitation of the enzyme occurred when concentrations of Woodward reagent higher than 1 mg/ml were used. Modified enzyme was prepared by adding 60 mg of Woodward reagent (Sigma Chemical Co., London) to a stirred solution of 1.2 g asparaginase in 60 ml 0.1 M sodium phosphate buffer, pH 7.4 at 0° for 4 hr. The product was dialysed and concentrated as described above.

Reaction with activated dextrans. Asparaginase was coupled to periodate activated dextrans by the following procedure. One gram of various soluble dextrans whose molecular weights ranged between 1.1×10^5 and 2×10^6 (obtainable from Pharmacia, Uppsala, Sweden) were dissolved in 15 ml water at room temperature. Sodium metaperiodate (3.5 g) was dissolved in 10 ml hot water which was then added to each dextran solution. The reaction vessel was incubated at 4° for 18 hr. The oxidized dextran was dialysed at room temperature against two 5 litre lots of water for 4 hr each and finally against 5 litres of 0.1 M sodium phosphate buffer pH 7.4 for 16 hr.

Five millilitres of a solution of asparaginase in the phosphate buffer (25 mg protein/ml) were added to the activated dextran and were allowed to react at 4° for 48 hr. The reaction was stopped by reducing the aldehyde groups by 1.2 ml freshly prepared sodium borohydride solution (50 mg/ml) and incubating the mixture for 1 hr at 4°. The product was dialysed and concentrated as described earlier.

Exclusion chromatography. Fine grade Sephadex G200 (Pharmacia Ltd., London) was swollen in 0.1 M sodium phosphate buffer, pH 7.4, by heating for 5 hr at 90–100°. Jacketed columns (90 cm long and 4.5 cm diameter; Wright Scientific Ltd., London) were packed with the swollen Sephadex under gravity and washed with the same buffer. After setting the flow rate at 18 ml/hr, the enzyme solution (approx. 5 ml) was loaded onto the gel. Effluent fractions (4 ml) were collected by an Ultrorac 7000 fraction collector (LKB, Sweden). The absorbance of each fraction was determined spectrophotometrically at 276 nm and 15μg protein samples from each fraction were analysed by the disc electrophoresis apparatus described below. Selected fractions were pooled to provide solutions of monomeric, dimeric and higher polymeric derivatives of the enzyme. These solutions were concentrated by pressure dialysis as described earlier and dialysed against a solution (2 litres) containing 0.11 M NaCl and 2% (w/v) glucose for 18 hr at 4° before being freeze dried in vials. The vials, each containing about 10 mg protein were stored at 4°.

Polyacrylamide gel electrophoresis. The method used was based on those described by Ornstein [11] and Davis [12]. The reagents used were electrophoresis grade material from BDH Ltd., Poole, Dorset. The gels (5 mm diameter) containing 7.5% (w/v) acrylamide and 0.2% (w/v) *NN'*methylene-bis-acrylamide were polymerized with 0.16% (w/v) ammonium persulphate and 0.75% (w/v) *N,N,N',N'*-tetramethylene diamine. Each sample containing about 15 mg protein was applied to the gel in about 10μl solution; the protein was diluted to this concentration with the running buffer solution saturated with sucrose. The running buffer (pH 4.5) contained 6.5 ml glacial acetic acid and 31.2 g β-alanine made up to a litre with water. Electrophoresis was performed at 2.5 mA per gel on a Shandon Disc Electrophoresis apparatus, Mark III (Shandon Southern Instruments Ltd., Camberley, Surrey). The gels were removed and stained with a solution of 0.35% (w/v) naphthalene black (BDH Ltd., Poole, Dorset) in a methanol : acetic acid : water solvent mixture (30 : 10 : 60 by vol.) for 1 hr and then destained with 7% (v/v) acetic acid. The gels were scanned with a Joyce–Loebl Chromoscan and the relative concentrations of the variously sized species were determined from the densitometer tracings and by using the integration facility of the instrument.

Sodium dodecyl sulphate–polyacrylamide gel electophoresis. Gels were prepared from 7.5% (w/v) acrylamide as described by Weber and Osborn [13] with a running medium of 0.1 M sodium phosphate buffer, pH 7.4, containing 1% (w/v) sodium dodecyl sulphate (SDS) (BDH Ltd., Poole, Dorset). The protein samples were incubated in this buffer for one minute at 100° before applying about 15 mg protein in 10μl solution saturated with sucrose. After electrophoresis on the Shandon Mark III apparatus at 6 mA per gel for 4 hr the gels were recovered and treated as described above.

Isoelectric focusing. The method described by Robinson [14] was used. The ampholines were incorporated into the gel mixture before polymerization to give a pH range of 3.5 to 10. Samples (about 150μg protein) were applied to the gel midway between the anode and cathode. The apparatus was switched on for 24 hr with the power not exceeding 1 W and the temperature maintained at 5°.

Protein concentration and asparaginase activity. These were determined by automatic colorimetric methods [15].

Amino acid analysis. Samples treated as described below were hydrolysed with 6 M–HCl in sealed tubes under nitrogen for 22 hr at 115° and the hydrolysates analysed on a Technicon Autoanalyser.

The estimation of the degree of substitution of lysine groups following the various chemical treatments of the enzyme cannot be determined by direct analysis because the substituted lysine groups may be hydrolysed by the acid and reappear as free lysines. To avoid this the unmodified enzyme and the different forms of modified enzyme were treated with 2,4-dinitrofluorobenzene to react with the unsubstituted lysine groups [16]. In this procedure 0.1 ml 2,4-dinitrofluorobenzene (Koch Light Labs., Colnbrook, Bucks) and 0.2 g sodium bicarbonate were added to 0.5 ml solution containing enzyme which had been denatured in 80% ethanol for 18 hr. Unsubstituted lysine appeared as ε-DNP lysine after acid hydrolysis and this was eluted from the

amino-acid analyser column after arginine. A comparison of the areas under the peaks produced by hydrolysates and known concentrations of ε-N-DNP lysine (Sigma Chemical Co., St. Louis, USA), enabled the number of lysine groups in native and modified enzymes to be determined.

Incorporation of ^{125}I *into asparaginase and its derivatives.* The method was based on that used for the iodination of hormones [17]. To the enzyme solution in saline phosphate buffer (2 mg protein in 50 μl 0.11 M-NaCl in 0.1 M sodium phosphate buffer at pH 7.4) 5 μl of Na ^{125}I solution 0.5 mCi; (Radiochemical Centre, Amersham, Bucks) and 20 μl of Chloramine T (0.01 M in saline phosphate buffer) were added. After 3 min at about 20°, 20 μl of 0.01 M-sodium metabisulphate and 40 μl of 1% (w/v) potassium iodide were added in that order. The mixture was filtered through a column (30 cm long and 1.2 cm diameter) containing Sephadex G25 (Pharmacia Ltd., London) which had been equilibrated against saline phosphate buffer. The enzyme was displaced with the same buffer and collected in about 5 fractions (0.5 ml each) which were pooled and stored at −20° for not more than 2 days before use.

Biological tests. The stored freeze-dried asparaginase and its derivatives were reconstituted in physiological saline (Polyfusor grade saline obtainable from Boots Co. Ltd., Nottingham) less than an hour before use. About 2000 units of asparaginase were injected intravenously into cross-bred rabbits weighing between 2.5 and 3.6 kg (obtained from Allington Farm, CDE, Porton Down, Nr. Salisbury). About 40 units of asparaginase were injected intraperitoneally into pathogen-free C3H mice (Olac Southern Ltd., Bicester, Oxford). Blood samples taken at regular intervals over a 48 hr period were delivered into heparinized tubes and the asparaginase activity was determined immediately.

The radioactivity of labelled enzyme was measured in vials containing one ml of whole blood or plasma. The vials were placed in the centre of a well type sodium iodide scintillation counter (Isotope Developments Ltd., Aldermaston) and the c.p.s. were determined.

RESULTS

Reaction between asparaginase and dimethylsuberimidate. The formation of cross linkages was demonstrated by the SDS-polyacrylamide gel electrophoresis of reacted and unreacted enzyme samples. The unreacted enzyme yielded a single stained band of dissociated protomers whereas the reacted enzyme preparation also showed multiple bands of various cross-linked protomers with higher molecular weights.

The inactivation of asparaginase as a function of dimethylsuberimidate concentration was similar whether enzyme concentrations of 4, 10 or 20 mg/ml were used (Fig. 1); precipitation occurs readily at higher concentrations of enzyme and dimethylsuberimidate. The effect of reagent concentration on the substitution of the lysine groups of the enzyme and on the polymerization of the enzyme are also shown on Fig. 1. The substitution increases as the ratio of enzyme : reagent concentration decreases and the polymer content increases as the ratio of enzyme : reagent concentration increases.

A closer examination of the reaction showed that the courses of inactivation, substitution of lysine groups and polymerization of the enzyme did not follow one another exactly (Fig. 2). Inactivation and polymerization of the enzyme continued throughout the 24 hr period whereas most of the substitution occurred in the first 2 hr of reaction. It is likely that the initial substitutions were intramolecular linkages formed between suitably spaced pairs of lysine groups. The rate of substitution decreased during the reactions as fewer such pairs were available and the rate decreased further when only intermolecular linkages were eventually possible; the rate of formation of intermolecular linkages was dependent on the concentration of protein in the solution. Even in large excess of dimethylsuberimidate (molecular ratio dimethylsuberimidate : asparaginase 660 : 1) only 50 of the 72 lysine groups in each

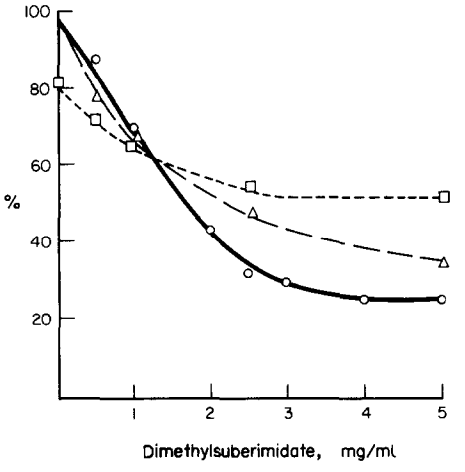

Fig. 1. The effect of dimethylsuberimidate on asparaginase (10 mg/ml) in 0.2 M triethanolamine hydrochloride buffer, pH 8.5, at 0° for 4 hr with respect to percentage enzyme activity (○), percentage unsubstituted lysine groups in enzyme (△), and percentage monomeric enzyme remaining in the reaction mixture (□).

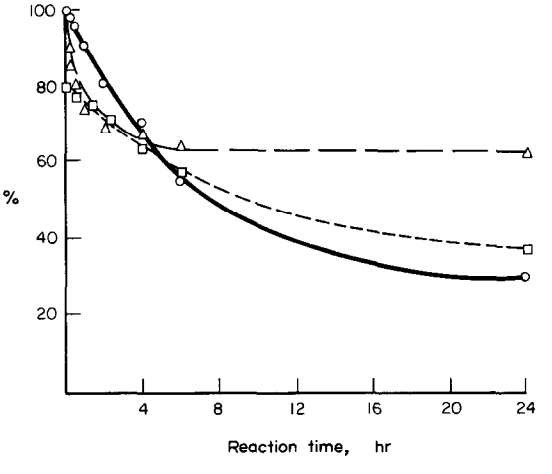

Fig. 2. The effect of dimethylsuberimidate (1 mg/ml) on asparaginase (10 mg/ml) in 0.2 M triethanolamine hydrochloride buffer, pH 8.5, at 0° with respect to percentage enzyme activity (○), percentage unsubstituted lysine groups (△), and percentage monomeric enzyme remaining in the reaction mixture (□).

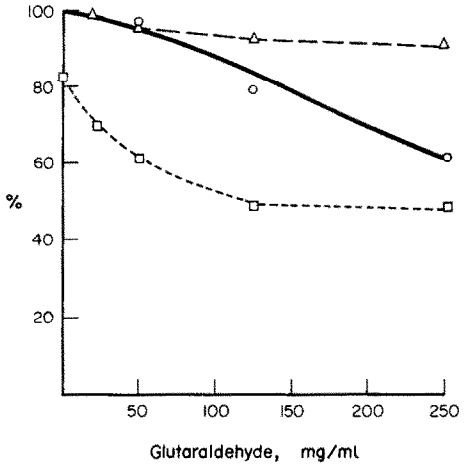

Fig. 3. The effect of glutaraldehyde on asparaginase (24 mg/ml) at 20° in phosphate buffer, pH 7.4, for 4 hr with respect to percentage enzyme activity (○), percentage unsubstituted lysine groups (△), and percentage monomeric enzyme remaining in reaction mixture (□).

asparaginase molecule were substituted (i.e. about 69 per cent of the total). Presumably, the other lysine groups were inaccessible to this reagent.

Reaction between asparaginase and glutaraldehyde. Glutaraldehyde was more effective in forming higher molecular weight polymers of asparaginase without inactivating the enzyme. In contrast to the dimethylsuberimidate reaction inactivation increased by increasing both the glutaraldehyde and protein concentration and significant polymerization was achieved with the substitution of only a few lysine groups (Fig. 3). Considerably fewer intramolecular covalent bonds appeared to be formed in this case. The soluble derivatives of glutaraldehyde contained only 3 to 4 substituted lysine groups per asparaginase molecule. Further substitution of the molecule by increasing the reagent concentration caused the enzyme to precipitate very rapidly.

Reaction between asparaginase and Woodward reagent K. The effect of the concentration of this reagent on enzyme activity is shown in Fig. 4. This reaction has

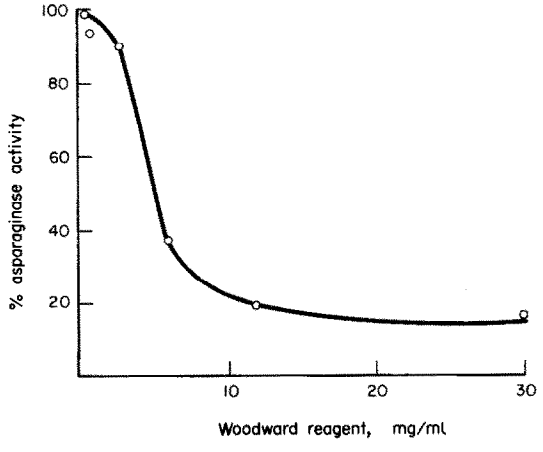

Fig. 4. The effect of Woodward reagent on asparaginase activity. The reaction was with the enzyme (10 mg/ml) in phosphate buffer, pH 7.4, at 0° for 4 hr.

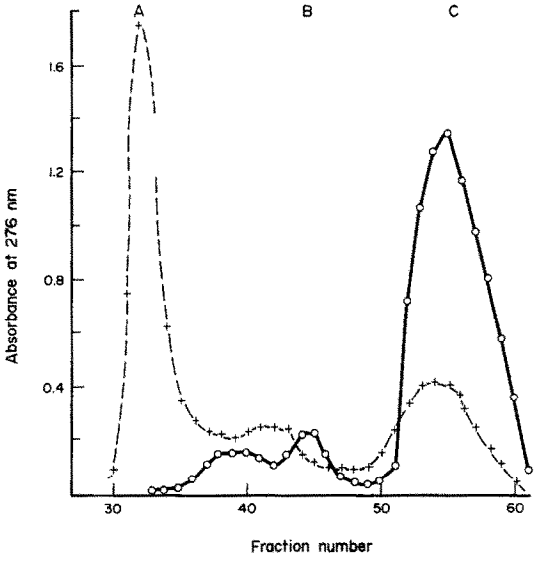

Fig. 5(a). Exclusion chromatography of asparaginase and its derivatives on Sephadex G200. Unmodified asparaginase (○) and products of reaction between asparaginase and dimethylsuberimidate as described in Methods . . . (+). Analyses by polyacrylamide gel electrophoresis were carried out on samples (15 μg protein) of fractions. Following this analysis the following fractions containing derivatives of the reaction ie. 31–33 (higher polymers) 42–44 (dimers and trimers) and 52–60 (monomers) were pooled, concentrated and dialysed and denoted as D/A, D/B or D/C (Tables 1 and 2) respectively in subsequent biological tests.

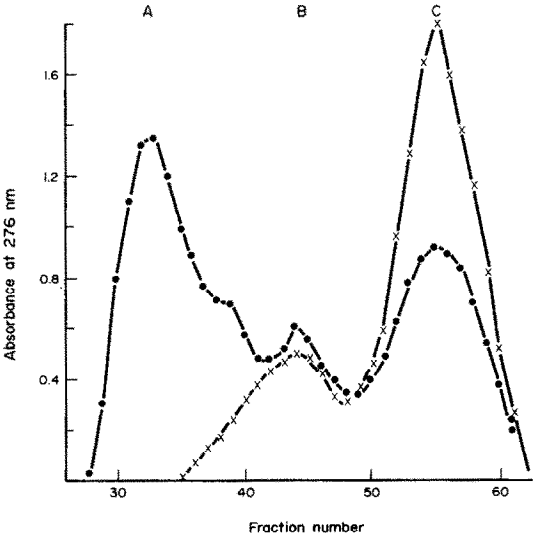

Fig. 5(b). Exclusion chromatography of gluteraldehyde (○) and Woodward reagent (X) derivatives of asparaginase prepared as described in Methods. After analysis as described in (a) fractions 29–34, 44–46 and 52–60 of gluteraldehyde derivatives and fractions 40–46 and 52–60 of Woodward derivatives were pooled, concentrated and dialysed and were denoted as G/A, G/B, G/C, W/B and W/C respectively (Tables 1 and 2).

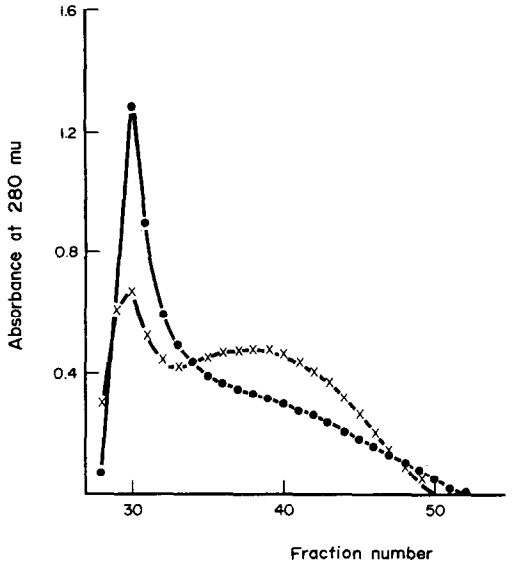

Fig. 5(c). Exclusion chromatography of Dextran 2000 – (●) and Dextran 110– (×) derivatives of asparaginase prepared as described in Methods. Fractions 29–40 were pooled, concentrated and dialysed. The Dextran 2000-asparaginase is denoted X (2000)/A and the Dextran 110-asparaginase is denoted X (110)/A.

not been studied in great detail because a relatively low yield of soluble polymers of the enzyme was obtained in exploratory tests.

Reaction between asparaginase and dextrans. Dextran dialdehyde is formed by oxidising dextran with sodium periodate. The dialdehyde is a linear polymer containing two aldehyde groups per monosaccharide unit. Dextrans of molecular weight 1.1×10^5, 2.5×10^5, 4.5×10^5 and 2.0×10^6 were activated in this case. It was found that 17 of the lysine groups in each enzyme molecule were substituted to form Schiff bases which are subsequently reduced to stable secondary amines by sodium borohydride. It was shown by SDS-polyacrylamide gel electrophoresis and exclusion chromatography that very large molecules are produced by this reaction. Under the conditions described in this paper about 30 per cent of the enzyme activity remains after the chemical treatments with each of the activated dextrans.

Preparation and properties of asparaginase derivates used in biological tests. The elution profiles following exclusion chromatography of the mixture following reaction with dimethylsuberimidate, glutaraldehyde and Woodward reagent can generally be divided into three main sections (Fig. 5); (C) includes monomeric derivatives of asparaginase, (B) the dimeric and trimeric forms and (A) the highly polymeric derivatives (molecular weight of 5.2×10^5 and over). The same percentage of lysine groups was substituted in (A), (B) and (C) derivatives with dimethylsuberimidate (Table 1). This should be expected because all forms contain the same number of intramolecular bonds and only a small ratio of the substituted lysine need be involved in intermolecular linkages that produce the high polymers. In addition, some lysine groups are modified to $-NH-C(=NH_2)[CH_2]_6COOCH_3$ groups which are formed by hydrolysis of one of the diimidate groups either before or, more likely, after the other had reacted with the enzyme.

It is shown that only a small amount of soluble higher polymers of asparaginase is obtained following modification with the Woodward reagent (Fig. 5). On the other hand all the enzyme appears to have coupled to the activated dextrans to form polymers of very high molecular weights (Fig. 5).

Compared to the native enzyme which had a fairly well defined isoelectric point (pI) between 8.30 and 8.35, each of the modified preparations were relatively heterogeneous in this respect (Table 1). The shift in pI was small in each of the derivatives tested; the largest shift was caused by reacting the enzyme with glutaraldehyde.

Persistence of asparaginase and its derivatives in rabbits and mice. The clearance of unmodified and modified asparaginase from the circulating blood of rabbits is illustrated in Fig. 6. The clearance of unmodified *E. carotovora* asparaginase from rabbits obeyed the kinetics of a first order reaction with a half-life of 11 hr; the clearance of the modified enzyme from rabbits did not always follow first order kinetics and this made

Table 1. Properties of L-asparaginase and its derivatives used in biological tests

Sample	Specific activity U/mg		'Free' lysines per enzyme molecule *	% Protein present as:- monomer	dimer	trimer	higher polymers
Unmodified L-asparaginase	550	8.30 – 8.35	72	81	16	3	0
D/A+	370	8.2 – 8.1	45	0	0	0	100
D/B+	413	8.3 – 8.1	46	0	89	11	0
D/C+	408	8.3 – 7.8	47	96	4	0	0
G/A+	536	7.6 – 7.5	67	0	0	0	100
G/B+	535	7.6 – 7.5	68	2	73	15	10
G/C+	534	8.1 – 7.5	69	89	11	0	0
W/B+	515	8.1 – 7.5	ND	20	63	14	0
W/C+	538	8.3 – 7.5	ND	86	12	2	0
X(2000)/A+	198	ND	17	0	0	0	100
X(110)/A+	188	ND	17	0	0	0	100

* Enzyme molecular weight equals 135,000.
+ All these prefixes are defined in Fig. 6.

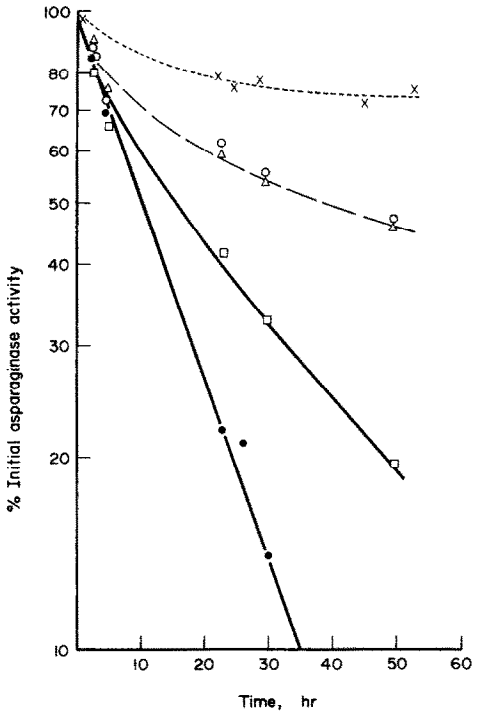

Fig. 6. Clearance of asparaginase activity from circulating blood of rabbits. Unmodified enzyme (●), fractions from the dimethylsuberimidate modified enzyme : D/A . . . (○); D/B . . . (△); D/C . . . (□), fractions of dextran modified enzyme X(110) /A . . . (X). The initial activity in the blood was determined by sampling 1 hr after intravenous injection at 2000 units enzyme.

quantitative measurement difficult. Therefore, to compare the clearance of these forms a method of measurement introduced by Putter [18] was used: as the time required for the enzyme activity in the circulating blood to decrease to one-half of its initial value (the initial activity was taken at 1 hr after intravenous injection of the material) (Table 2). This is a valid method since the progess curve for enzyme concentration vs time was always independent of the dose of enzyme injected into the animal. Characteristically, the decay of enzyme activity of the modified form initially was relatively fast but decreased considerably after a few hours (Fig. 6). The larger molecular derivatives, particularly the dextran linked enzymes, persist much better than the unmodified enzyme. It is also noted that the monomeric derivatives persist better than the unmodified enzyme.

Table 2. Time for enzyme activities of asparaginase and its derivatives to be reduced to one-half its initial activity after intravenous injection into rabbits

Sample	Time required for enzyme activity to be reduced to one-half its initial activity after intravenous injection
Unmodified asparaginase	11 ± 1
D/A	39 ± 3
D/B	39 ± 3
D/C	16 ± 2
G/A	27 ± 2
G/B	27 ± 2
G/C	21 ± 2
W/B	21 ± 2
W/C	15 ± 2
X(2000)/A	190 ± 20
X(110)/A	190 ± 20

In the small number of tests carried out in mice the dimethylsuberimidate modified dimers of asparaginase persisted for considerably longer periods in the blood than the native enzyme. The half-lives were more readily determined in this animal because the clearance of both modified and native enzymes, injected intraperitoneally, followed first order kinetics. The half-life of the modified enzyme was 28 hr compared with 7.5 hr for the unmodified enzyme.

Experiments with ^{125}I*-labelled enzyme.* Further experiments were designed to show whether the differences in losses of enzyme activity from blood could be attributed to differences in their rates of removal, or to inactivation, or to both. The similar diminution of radioactivity and enzyme activity of the labelled asparaginase and labelled dimethylsuberimidate modified asparaginase (Fig. 7) indicates that the improved persistence of the modified enzyme cannot be attributed merely to the inhibition of some process that inactivates the enzyme. The results show, in fact, that both unmodified and modified enzymes are removed from the circulation intact.

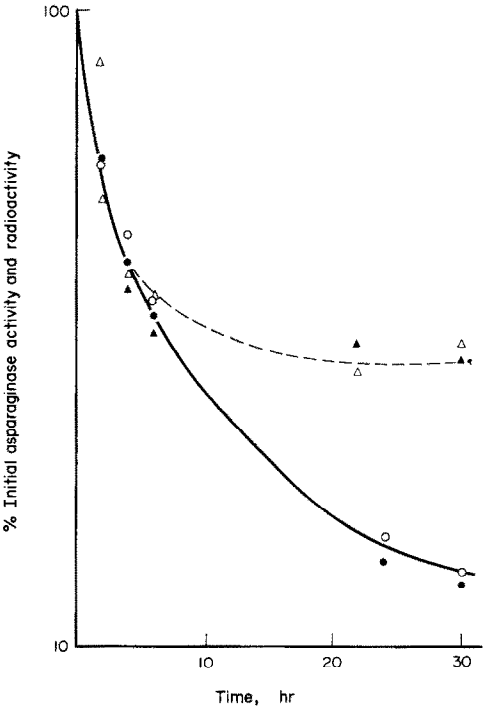

Fig. 7. Clearance of radioactive asparaginase from the circulating blood of rabbits. ^{125}I-labelled enzyme (enzyme activity and radioactivity denoted by ○ and ● respectively) and ^{125}I-labelled dimethylsuberimidate modified fraction, D/B (enzyme activity and radioactivity denoted by △ and ▲ respectively) were injected intravenously into the rabbits. Initial values of enzyme and radioactivity in the blood were determined at 1 hr.

DISCUSSION

The radioactivity experiments showed that the enzymes may be removed intact from the circulating blood. However, the mechanism by which foreign enzymes like microbial asparaginase are eliminated from the circulation is not understood at the molecular level. Such knowledge could prove useful for improving the therapeutic effectiveness of such materials. Some attempts have been made to prevent the rapid clearance of asparaginases. One type of approach involved physically entrapping the enzyme in liposomes [19, 20] or in red blood cells ghosts [21]. Another approach involved chemical modification of the enzyme because evidence has been presented that the persistence of foreign protein can be altered following treatment such as acetylation, glycosylation, deamination and amidination [4, 22–26].

The use of bi- and polyfunctional chemical reagents to produce polymers of asparaginase show that there is a relationship between the molecular weight and the persistence of the enzyme. The largest polymers produced by binding the enzyme to various activated dextrans have been shown to persist extremely well. It is expected that because of this increased persistence of the modified enzyme, the antitumour potency will markedly increase. Because of this therapeutic potential the use of toxic chemicals for modification of the enzymes has been avoided. Therefore, dextrans activated by cyanogen bromide and the bifunctional compound dinitrofluorobenzene have not been used although these compounds are known to produce enzyme derivatives with improved persistence in blood ([26], and unpublished observations).

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