9
Biomedical Applications of Enzymes and their Polyethylene Glycol Adducts

GUY FORTIER

Laboratoire d'Enzymologie Appliquée, Groupe de recherche en Biothérapeutique Moléculaire, Département de Chimie-Biochimie, Université du Québec à Montréal, Montréal (Québec), Canada

Introduction

The field involving the application of enzymes is expanding every day. We now find that enzymes are used in many branches of our economy (food, textiles, pulp and paper, electronics, medicine, chemistry and so forth), mainly as tools for specific biotransformations, for diagnostic and quality control, and as pharmaceutical drugs.

To develop and optimize the use of enzymes in the next decade, many problems have to be resolved. They concern:

1. the instability of the structure and the activity of the enzyme under various operational conditions;
2. the availability of the enzyme at a reasonable cost;
3. the obtention of a very specific biotransformation capacity; and
4. the far too rapid clearance of the enzyme in biological fluids.

Many research groups are focusing on these concerns, and approaches such as genetic and protein engineering, as well as engineering of the microenvironment of the enzyme, are presently privileged solutions. These points have been reviewed recently by many authors (Mozhaev and Martinek, 1990; Gianfreda and Scarfi, 1991; Gupta, 1991; Mattiasson and Adlercreutz, 1991; Roig and Kennedy, 1992; Janecek, 1993; Mozhaev, 1993). An enzyme should be considered as a natural 'green product' which is fully biodegradable and safe for the environment. It is easy to believe that the enzyme will become a very attractive tool in the future as the tendency of consumers to request more natural and biodegradable products and processes will be enforced.

Abbreviations: ADA, adenosine deaminase; Ado, adenosine; ALL, acute lymphoblastic leukaemia; AML, acute myeloblastic leukaemia; ASNase, asparaginase; dAdo, 2'-deoxyadenosine; dATP, deoxyadenosine triphosphate; BSA, bovine serum albumin; HRP, horseradish peroxidase; mPEG, monomethoxy polyethylene glycol; NMR, nuclear magnetic resonance; OPA, *o*-phthaldehyde; PEG, polyethylene glycol; rIL-2, recombinant interleukin-2; SDS/PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TNBS, trinitrobenzenesulphonic acid.

Biotechnology and Genetic Engineering Reviews — Vol. 12, December 1994
0264–8725/94/12/329–356 $20.00 + $0.00 © Intercept Ltd, P.O. Box 716, Andover, Hampshire SP10 1YG, UK

Table 1. Partial list of enzymes in clinical use as potential or established treatments

Disease	Enzyme deficiency	Substrate accumulation	Potential therapeutic enzyme	Specific site to be targeted	[a] Pegylated trial
Experimental treatments for storage diseases					
Aspartylglycosaminuria	Glycosylasparaginase	Aspartylglucosamine	Amidase	Lysosome	
Fabry	α-Galactosidase	Ceramide	α-Galactosidase	Lysosome	1
Gaucher	β-Glucocerebrosidase	Glucocerebroside	β-Glucocerebrosidase from placenta	Liver, spleen, bone mannose receptor, macrophage	
Mucopolysaccharidosis	β-Glucuronidase β-Glucuronidase	Mucopolysaccharide			
Pompe	α-1,4 Glucosidase	Intracellular glycogen	α-1,4 Glucosidase	Mannose cell receptors	
Wolman	Lipase, cholesteryl esterase	Cholesteryl ester	Cholesteryl esterase	Fibroblast receptor insulin or apoB	
Experimental treatments for metabolic diseases					
Acatalasaemia		Oxygen free-radical	Catalase	Systemic	2
Ascorbic acid deficiency	Gulonolactone oxidase		Gulonolactone oxidase	Systemic	3
Hyperargininaemia	Arginase	Arginine	Arginase	Systemic	4,5
Hypophosphatasia	Alkaline phosphatase		Alkaline phosphatase		
Phenylketonuria	Phenylalanine hydroxylase	Phenylalanine	Phenylalanine ammonia lyase	Systemic	6,7
Severe combined immunodeficiency	Adenosine deaminase	Deoxyadenosine homocysteine	Adenosine deaminase bovine	Equilibrium between cells and blood	8,9
Experimental treatment for organ failure					
Gout		Uric acid	Uricase	Systemic	10
Jaundice	Liver disease	Bilirubin conjugates bilirubin	Bilirubin oxidase UDP glucuronyl transferase	Systemic Systemic	11
Liver failure		Phenol and tyrosine	Tyrosinase	Systemic	
Renal failure		Urea	Urease	Systemic	

Experimental treatments for traumatic and acute injuries					
Inflammatory	Oxygen free-radical	Superoxide dismutase	Systemic	12	
Ischaemia reperfusion	Oxygen radical	Catalase	Systemic or local		13
			Superoxide dismutase	Systemic or local	14
			Ceruloplasmin	Systemic or local	
Thromboemboly		Clot	Fibrinolysin	Fibrin	
			Streptokinase	Plasminogen activation	15
			Urokinase	Plasminogen activator	16
			Batroxobin	Defibrinogenation	17
Other enzymatic treatments					
Burn treatment		Dead tissue	Collagenase	Local	
Various cancers			Amine oxidase		18
			Arginase		19
			Asparaginase	Systemic or local	
			Asparaginase–glutaminase		
			Glucose oxidase		
			Ribonuclease		
Clot formation		Heparin	Heparinase	Systemic	
Disc problem			Chymopapain	Local	
Herniated disc			Collagenase	Local	
Viral infection			Ribonuclease	Systemic	20

[a] Reference relative to the modified PEG enzyme: 1, produced by Enzon Co.; 2, Davis *et al.* (1980); 3, Hadley and Sato (1989); 4, Davis *et al.* (1980); 5, Savoca *et al.* (1984); 6, Davis *et al.* (1980); 7, Wieder *et al.* (1979); 8, Hershfield *et al.* (1987), Levy *et al.* (1988), Hershfield, Chaffee and Sorensen (1993); 9, Beauchamp, Daddona and Menapace (1984); 10, Nishimura *et al.* (1979), Davis *et al.* (1980), Nishimura, Matsushima and Inada (1981); 11, Maeda, Seymour and Miyamoto (1992); 12, Davis *et al.* (1980), Greenwald (1990); 13, Muizelaar *et al.* (1993); 14, Lehman *et al.* (1992), Tang *et al.* (1992), Thibeault, Rezaiekhaligh and Mabry (1993); 15, Fears *et al.* (1992); 16, Garman and Kalindjian (1987); 17, Nishimura *et al.* (1983); 18, Savoca *et al.* (1979, 1984); 19, see *Table 4*; 20, Laznicek *et al.* (1993).

This chapter will focus on the current biomedical applications of enzymes and their polyethylene glycol adducts, as well as potential future developments.

ENZYMES AS PHARMACEUTICAL DRUGS

For a long time in medicine, enzymes have been used as diagnostic tools (Boehringer Mannheim, 1998–89), as prognostic tools and as disease markers (Guilbault, 1983; Schwartz, 1992). More recently, enzymes have found applications as a new class of therapeutic agents (Klausner, 1983; Klein and Langer, 1986; Goldberg, 1992; Maeda *et al.*, 1992; Smith *et al.*, 1993). This new emerging field, named Enzymotherapy, should rapidly expand in the next decade when problems related to their use will be solved. Enzymes may well supply a new class of very important biological drugs. *Table 1* gives a non-exhaustive list of enzymes currently in clinical use, as well as future prospects as established treatments. For the sake of clarity, *Table 1* is subdivided on the basis of the main area in which enzymes can be useful, such as:

1. agents for replacement therapy in inherited genetic and metabolic diseases;
2. scavengers to limit the tissue damage during the course of inflammatory diseases and various ischaemia/reperfusion injuries;
3. specific detoxification agents during acute chemical poisoning;
4. temporary palliative agents during an acute organ failure;
5. agents for corrective treatments; and
6. antineoplastic agents.

Points 1 and 6 will be discussed in further detail in this chapter, using three case examples.

It is well known that several major problems limit the use and decrease the efficiency of enzymes in the clinic. One of these is the immunological reaction frequently observed following multiple injections of foreign enzymes, which can lead to antibody formation that neutralizes the enzyme or that can also lead to a severe anaphylactic shock. In addition, the half-life of the majority of enzymes in the bloodstream is impaired by rapid glomerular filtration and non-specific absorption, by the endothelial system and by the endoplasmic reticulum. The lack of specificity in targeting the enzyme to 'selected' cells or organs requires that higher doses of enzyme need to be used, directly affecting the cost of the treatment. Therefore, to overcome these limiting factors, which are acting against the expansion of the use of enzymes in humans, an approach consisting of the surface modification of clinically useful enzymes was proposed. These enzymes would be grafted with biocompatible polymer chains, with the aim of diminishing their immunogenicity and increasing their plasma half-life. During the past 20 years this has been considered as the best performing strategy, for which polyethylene glycol is the most successfully used and the most widely studied biocompatible polymer. Finally, to reduce the high cost associated with the use of pure enzymes in the treatment of patients, a solution should be found by establishing better multidisciplinary collaboration between enzymologists, clinicians and genetic engineers, that will allow the design of enzymes with both improved activity and targeting capacity.

Polyethylene glycol adducts

Many types of polymers have been tested for this purpose but the monomethoxy polyethylene glycol of molecular mass of 5000 gives the most spectacular results in terms of increasing the biological half-life of the enzyme in blood and limiting the immunological response. *Table 2* describes the changes in the characteristics of macromolecules when they are covalently modified by PEG. Some most noteworthy examples of these positive changes are:

1. the increase in water solubility, which is not a major problem with protein;
2. the increase in the resistance of the modified protein to protease action, which is of great interest for the lack of antibody response;
3. the decrease of immunogenicity due to masking of the antigenic sites of the protein by the polymer chains; and, finally,
4. the modification of its biochemical behaviour and its pharmacokinetics.

Table 2. Change in the characteristics of molecules or macromolecules modified with covalently bound polyethylene glycol

What is increased?	What is decreased?	What else?
Water solubility	Immunogenicity	Antigenicity is masked
Resistance against proteases	Toxicity	Pharmacokinetics are modified
Cellular influx	Blood clearance	
Affinity to the cellular membrane	Fouling	
Chemical and physical stability		
Biocompatibility		
Hydrodynamic volume		

CHEMISTRY OF PEG

In order to achieve the surface derivatization of an enzyme by covalent addition of polymer chains, many chemical procedures have been developed to obtain polyethylene glycol (PEG) and monomethoxy polyethylene glycol (mPEG) with reactive functional groups at their extremities. The chemistry of these functional groups, which can react with free amino, carboxylic or sulphydryl groups of the amino acids at the surface of an enzyme under mild aqueous conditions, has been reviewed recently (Harris, 1985; Harris *et al.*, 1992). The functionalization of PEG with the aim of specific cross-linking reactions with side-chains of different amino acids has been discussed in detail (Zalipsky and Lee, 1992). As an example of a reagent specific for amino acid side-chain residues, the formation of a PEG-thiol protected with a 4-thiopyridone derivative (Woghiren, Sharma and Stein, 1993) allows specific derivatization of cysteine residues of the protein.

The covalent reaction or attachment of a reactive group to the hydroxyl functions of PEG was successfully achieved with 1,1'-carbonyl diimidazole (Beauchamp *et al.*, 1983), cyanuric chloride (Nishimura *et al.*, 1983; Jackson *et al.*, 1987), 2,4,5-trichlorophenyl chloroformate or 4-nitrophenyl chloroformate (Veronese *et al.*, 1985; Fortier and Laliberté, 1993) with or without a peptide spacer arm of various lengths (Sartore *et al.*, 1991a), the Moffatt–Swern reaction (Wirth *et al.*, 1991), tresyl

chloride (Delgado *et al.*, 1990), and by various *N*-hydroxysuccinimide derivatives (Anderson and Tomasi, 1988; Zalipsky, Seltzer and Nho, 1991), such as succinimidyl active ester (Knauf *et al.*, 1988) and succinimidyl carbonate (Zalipsky, Seltzer and Menon-Rudolph, 1992) or with 4-fluoro-3-nitrobenzoic acid (Ladd and Snow, 1993). The advantage of using 4-fluoro-3-nitrobenzoic acid is that the addition of PEG on to the protein surface can be followed quantitatively.

Recently, the pegylation of peptides during their solid-phase synthesis has been performed successfully (Lu and Felix, 1993). It should also be mentioned that PEG addition is not only limited to enzymes and proteins. Various small drug-PEG adducts such as doxorubicin (Caliceti *et al.*, 1993), vitamin E (Sokol *et al.*, 1993), acetominophen and other drugs (Zalipsky, Gilon and Zilkha, 1983), and various peptide-PEG adducts such as interleukin-2 (Katre, Knauf and Laird, 1987; Balemans *et al.*, 1993; Ofosu-Appiah *et al.*, 1993), hirudin (Zawilska *et al.*, 1993) and granulocyte–macrophage colony-stimulating factor (Knüsli *et al.*, 1992), have been synthesized with success.

SEPARATION AND CHARACTERIZATION OF PROTEIN–PEG ADDUCTS

One of the most difficult tasks of PEG technology is related to the determination of the number of PEG chains added on to the surface of the proteins or enzymes and to the purification of the derivatized enzymes. This should represent a crucial step for the approval of the innocuity of the product by competent authorities. For the characterization of the preparation, several techniques were developed to evaluate the molecular weight of the protein–PEG adduct, with the aim of deducing the number of PEG chains *per* protein. Gel filtration using aqueous buffer, with or without organic solvent modifiers, as well as SDS/PAGE electrophoresis failed in the majority of cases to determine the molecular weight of the preparation. Explanations such as the entanglement of PEG in the PAGE matrix (Abuchowski *et al.*, 1977; McGoff, Baziotis and Maskewicz, 1988) and insulation of the protein from the electric field by PEG (Bode, 1976), as well as a dramatic increase in the hydrodynamic volume of the modified protein or enzyme, have been considered responsible for the lack of electrophoretic mobility in SDS/PAGE electrophoresis and for the increase of the observed resident time during gel filtration of PEG-modified protein.

A denaturing buffer composed of 8 M urea was used for gel filtration chromatography of various horseradish peroxidase PEG adducts (Fortier and Laliberté, 1993). The aim was to decrease the availability of water and to reduce hydrogen interactions, thus allowing the separation of the modified proteins. During the chromatographic analysis of eight different adducts of horseradish peroxidase (HRP) prepared with PEG of molecular masses varying between 750 and 35 000, it was observed that the peak width of preparations of HRP increased with the molecular mass of PEG adducts faster than predicted by the retention time. The increase of the peak width according to the molecular mass of PEG was explained in part by the heterogeneity of the commercial preparation of HRP used and by the polydispersity of the PEG, which is more marked with high molecular mass PEGs. This was in agreement with the conclusion of the experimental work of Kunitani *et al.* (1991) who showed that protein–PEG adducts should be regarded as a homogeneous distribution of molecular weights as a function of the polydispersity of PEG. Contamination of monomethoxy

polyethylene glycol by polyethylene glycol (Selisko, Delgado and Fisher, 1993) often leads to aggregation of the protein and to the misinterpretation of the molecular mass of the protein–polymer adduct.

It should be kept in mind that, due to its random coil structure, a polyethylene glycol with a molecular mass of 20 000 has a hydrodynamic volume equal to a protein of about 70 000 Da (based on its viscosity) (Tam and Tremblay, 1991). The increase in hydrodynamic volume has been well documented by a light-scattering study performed on native superoxide dismutase (SOD) and on modified SOD–mPEG$_{5000}$ (McGoff, Baziotis and Maskewicz, 1988). In this study, it was demonstrated that the apparent molecular diameter of native SOD increased from less than 3 nm to 13.2 nm when modified with mPEG with a molecular mass of 5000.

Separation of the PEG-modified protein from the unreacted PEG is a difficult task, mainly because PEG has a random coil structure which is well hydrated in aqueous solution (Bailey and Koleske, 1967). It has been demonstrated that ultrafiltration techniques can be useful for small molecular masses of PEG, but in the presence of high molecular masses of PEG (i.e. >5000) it is very difficult to separate unreacted PEG from protein-modified PEG, as was recently described in an extensive study by Lentsch and co-workers (Lentsch, Aimar and Orozco, 1993). They showed that, depending on the molecular mass of PEG in a bovine serum albumin (BSA) solution, it is difficult to increase the transmission of PEG through the ultrafiltration membrane without increasing the fouling of BSA at the membrane surface. They underlined the importance of a thick channel (thickness of the membrane) and a low cross-flow to enhance the PEG concentration polarization in order to eliminate PEG from the filtration solution.

One possible strategy which can enhance the ultrafiltration of PEG is based on the reduction of its hydrodynamic volume under conditions that are not detrimental to the activity of the modified enzyme. This can be obtained, as we have demonstrated (Fortier and Laliberté, 1993) by performing the ultrafiltration in the presence of 0.65 M potassium sulphate which is a kosmotrope salt (Chicz and Regnier, 1990), leading to a reduction in the viscosity of the PEG solution (Bailey and Koleske, 1967). Sulphate is one of the best kosmotrope salts and it helps to stabilize the enzyme or protein. Its use results in the dehydration of the PEG coil which then collapses, leading to a reduction in its hydrodynamic volume. This filtration procedure was evaluated and it was shown that the presence of this salt enhanced the efficiency of the ultrafiltration process by a factor of 2 to 3 times. Under these conditions, the pressure and the flow of the ultrafiltration device remained constant throughout the experiment, and a membrane with a smaller molecular weight cut-off was used to avoid the loss of biological material.

To address the problem of determination of the number of chains of PEG added to the surface of a protein, several techniques have been developed during the past 3 years to replace the classical spectrophotometric assay based on the reaction of the free amino groups of the protein with trinitrobenzenesulphonic acid (TNBS assay) (Fields, 1971; Snyder and Sobocinski, 1975) or the spectrofluorometric assay using fluorescamine (Stocks *et al.*, 1986). One technique consists of the addition of an amino acid spacer, such as an unnatural amino acid such as norleucine, between the protein and the PEG. The quantity of norleucine found by amino acid analysis is proportional to the number of chains added to the protein surface (Sartore *et al.*,

1991b). These authors have also shown, using seven different proteins modified by PEG to various degrees ranging from 10 to 80%, that TNBS and OPA (*o*-phthaldehyde) procedures that allow quantification of free lysine amino groups at the surface of the protein overestimated the number of amino groups that reacted with PEG compared to the amount of norleucine detected. Recently, the use of monomethoxy polyethylene glycol esters of 4-fluoro-3-nitrobenzoic acid enabled the addition of PEG on to the protein surface to be followed quantitatively by fluorescence (Ladd and Snow, 1993). More sophisticated methods were also elaborated, such as analysis of the protein adducts by nuclear magnetic resonance (NMR) (Jackson *et al.*, 1987) or analysis by mass spectrometry (Vestling *et al.*, 1993).

FACTORS AFFECTING THE CATALYTIC ACTIVITY OF AN ENZYME DURING PEG DERIVATIZATION

Table 3 summarizes the factors that can affect the catalytic activity of a biological macromolecule submitted to surface derivatization using activated PEG. *Table 4* shows a compilation of various enzymes and different derivatization procedures along with the percentage modification and the residual activity of the various enzyme–PEG adduct preparations. One of the first problems encountered during the derivatization of an enzyme is to select the PEG reagent that will minimize the loss of residual activity versus the number of polymer chains added. Strong and non-specific reagents, such as the mPEG-triazine ether derivative obtained by activation of mPEG with cyanuric chloride, often lead to a majority of enzyme derivatives with low specific activities. Many examples are given in *Table 4*, such as adenosine deaminase which has a residual activity for adenosine of 28% when cyanuric chloride is used to pegylate 40% of the lysyl residues. This is compared to a residual activity of 78% when a carbonyldiimidazole reagent is used to modify more than 80% of the lysyl residues. For other enzymes, such as catalase or superoxide dismutase, varying results have been obtained with the same reagent for the same level of modification. These variations can be related to the purity of the reagent and to the conditions of the reaction. A classical example based on the derivatization of alkaline phosphatase (Yoshinga and Harris, 1989) to a similar percentage of modification by different reagents gave varying residual activities. In this example, the succinimidyl-succinate PEG derivative acts very well, giving 61–79% modification with a small loss of specific activity of less than 10%. This is far better than the result obtained with the cyanuric chloride–PEG derivative. It was shown with some reagents, such as tresyl-chloride or cyanuric chloride-activated PEG, that the PEG adduct can be reacted with different functional groups of the protein, leading to denaturation. It became very difficult, when using the trinitrobenzene sulphonic acid assay, to extrapolate the number of PEG chains added because the assay does not account for addition of PEG to carboxyls, sugars or tyrosine due to the strong reactivity of this PEG derivative.

Another factor is related to the availability and the number of functional groups in the protein and their positioning with respect to the substrate fixation site and to the catalytic site. This can explain why some proteins such as catalase and superoxide dismutase give high residual activity after extensive modification, while others, such as phenylalanine ammonia lyase and uricase, lose most of their catalytic activity with minimal modification.

Table 3. Factors that affect the activity of an enzyme during its modification by polyethylene glycol

1. The coupling reagent bound to the PEG that reacted with the protein, they range from mild type (phenylcarbonate) to strong type (cyanuric chloride)
2. The molecular mass of the PEG, from M_r 750 to 100 000
3. The number of functional groups of PEG (monomethoxy or not, induced some polymerization)
4. The number of chains of PEG added to the surface of the enzyme
5. The functional group of the enzyme that would be derivatized, such as SH, NH_2, COOH, sugars
6. The stability of the chemical link
7. The reaction conditions, i.e. temperature, pH, co-solvent, etc.

As the importance and the usefulness of PEG adducts has increased during the past decade, the chemistry of PEG activation has attracted the attention of many organic chemists. A variety of chemical reagents specific for different functional groups of a protein are now available, and their reactions can be carried out in very mild aqueous conditions that are not detrimental to enzyme activity. The number of chains as well as the length of the PEG chain added to the surface of an enzyme can modulate the specific activity as well as the specificity of the enzyme for its substrate. For example, horseradish peroxidase modified with 3–4 PEG chains shows an increase in its specific activity towards phenylenediamine (*Table 4*). However, enzymes such as batroxobin, ribonuclease, lysozyme, metalloprotease *Vibrio* and other proteases lose their capacities to transform and to hydrolyse their natural high molecular weight substrates. This is due to steric hindrance induced by the PEG chain around their catalytic site that does not allow the macromolecular substrate to reach the catalytic centre. In the majority of cases, these modified enzymes are still very active in the presence of small synthetic substrates.

Another important factor that should be taken in consideration when performing derivatization of proteins is the durability of the chemical link between the protein and the PEG. For an *in vivo* utilization, which requires a long circulation time of the modified enzyme, a stable bond between the enzyme and the PEG is needed. To achieve this, an urethane bond is preferable to an ester bond which is more labile, having a half-life in blood of about 8 hours. Zalipsky and Lee (1992) have discussed the subject in a recent review paper.

The derivatization of an enzyme with PEG should be optimized in order to obtain a modified enzyme with high activity and with specific properties such as non-immunogenicity, high serum residence time, decrease in susceptibility to proteolysis and so forth. The user should therefore take into account the stability of the protein in the buffer at the pH at which the reaction will be carried out, to the functional group of the protein that will be modified, as well as to the number of chains that should be added to it. There are not enough detailed studies on the role of the PEG chain-length (Laliberté, Gayet and Fortier, 1994), on the nature of the functional group to use, on the ideal degree of modification to reach, versus the molecular weight of the protein and the number of available NH_2 groups, to enable the establishment of a general procedure. Thus, each protein is a unique case for which the reaction, the percentage of modifications and the choice of reagent should be optimized in a specific manner in order to take advantage of this technology.

Table 4. Effect of PEG activation reagents and the percentage modification on the residual activity of various proteins

Protein	PEG M_r	PEG activation reagent	Residual activity (substrate)	% Modification	Purpose of the study	References
Adenosine deaminase	5000	Cyanuric chloride	28% (adenosine)	40%	Pharmacokinetics of modified enzyme	Davis *et al.* (1981)
Adenosine deaminase	5000	Carbonyldiimidazole	76% (adenosine)	85%	Pharmacokinetics of modified enzyme	Beauchamp, Daddona and Menapace (1984)
Alkaline phosphatase	5000	Cyanuric chloride	67% and 33% (*p*-nitrophenylphosphate)	62% and 88%	Evaluation of the cross-linker on of the enzyme	Yoshinga and Harris (1989)
	5000	Tresyl chloride	82% and 86%	73% and 77%		
	5000	Carbonyldiimidazole	91% and 77%	56% and 78%		
	5000	Succinimidyl-succinate	98% and 93%	61% and 79%		
Arginase	5000	Trichlorophenyl-chloroformate	90% (arginine)	60%	Biochemical and pharmacokinetic behaviours	Visco *et al.* (1987)
Arginase	5000	Cyanuric chloride	65% (arginine)	53%	Pharmacokinetics	Savoca *et al.* (1979)
Asparaginase *E. coli*	5000	Not indicated	52% (asparagine)	70%	Pharmacology of the modified enzyme	Park *et al.* (1981)
Asparaginase *E. coli*	5000	Cyanuric chloride	0.9% (asparagine)	79%	Comparative study with new comb-shaped PEG	Kodera *et al.* (1992)
Bacteriorhodopsin	5000	Succinimidyl carbonate	As the native (proton pumping)	1 PEG chain	Study of the functionality of modified protein membrane	Sirokman and Fasman (1993)
Batroxobin	5000	Cyanuric chloride	2.4% (clotting), 93% (esterolytic)	29%	Evaluation of immunoreactivity, thrombin-like protease activity and blood clearance	Nishimura *et al.* (1983)
Bilirubin oxidase	5000	*p*-Nitrophenyl chloroformate	40–60% (bilirubin)	11–12 PEG added	Review on jaundice treatment Miyamoto (1992)	Maeda, Seymourand
Catalase	5000	Cyanuric chloride	100%	60–70%	Evaluation of cellular uptake of PEG-enzymes	Beckman *et al.* (1988)
Catalase	5000	Cyanuric chloride	67%, 37% and 12% (H_2O_2)	21%, 46% and 55%	Activity in organic solvents	Takahashi *et al.* (1984a)
Cholesterol esterase	5000	Cyanuric chloride	High (cholesterol linoleate)	n.d.	Cholesterol ester synthesis in benzene	Mori, Nakata and Endo (1992)
β-Galactosidase	2000, 8000, 20 000	1,1-Carbonyldiimidazole	50% (*o*-nitrophenylgalacto-pyranoside) for the different preparations	77, 60 and 54%, respectively	Study of transgalactosylation in organic solvents	Beecher *et al.* (1990)
β-Glucuronidase	5000	Cyanuric chloride			Potential treatment of mucopoly-saccharidosis	Lisi *et al.* (1982)
Gulonolactone oxidase	5000	Succinimidyl succinate	74% (ascorbate formation)	47% decreased	Clearance and immunogenicity were not	Hadley and Sato (1989)
Haemoglobin	5000 acid	Benzene hexacarboxylic	Decrease in oxygen affinity	3 PEG chains	Evaluation of PEG-Hb for blood substitute (1991)	Dellacherie and Léonard
Horseradish peroxidase	750–35 000	*p*-Nitrophenyl chloroformate	110% to 75% (phenylenediamine)	65%	Effect of chain length of PEG on stability of HRP against denaturating agents (pH, T°, etc.)	Fortier and Laliberté (1993), Laliberté, Gayet and Fortier (1994)
Horseradish peroxidase	1900	Reductive alkylation	110% (phenylenediamine)	42%	Activity in organic solvents	Wirth *et al.* (1991)
	5000	Reductive alkylation	88% (phenylenediamine)	37%		
Horseradish peroxidase	5000	Cyanuric chloride	70% (phenylenediamine)	60%	Activity in organic solvents	Takahashi *et al.* (1984b)
Horseradish peroxidase	20 000	Dithioester	88% (*o*-dianisidine)	33%	Activity in organic solvents (1989)	Souppe and Urrutigoity
Lipase	5000	Cyanuric chloride	80% (esterolysis of triglyceride)	55%	Ester synthesis in organic solvents	Inada *et al.* (1984)

Lipase	5000	Cyanuric chloride	43% (hydrolytic activity)	49%	Esterification of chiral *s* alcohol in organic solvents	Kikkawa *et al.* (1989)
Metalloprotease *Vibrio*	5000	?	100% (Z–Gly–Phe), 20% (casein)	80%	Reduce the binding to δ-macroglobulin	Narukawa, Miyoshi and Shinoda (1993)
Ovalbumin	6400	Cyanuric chloride	–	0–14 PEG	Improve preparation and purification	Jackson *et al.* (1987)
Phenylalanine ammonia lyase	5000	Cyanuric chloride	40%	28%		Wieder *et al.* (1979)
Ribonuclease	5000	Hydroxysuccinimidyl ester	80% (cytidine-2,3 cyclic phosphate)	8–9 PEG chains	Evaluation of pharmacokinetics and distribution in rats	Laznicek *et al.* (1993)
Ribonuclease	1900	Trichlorophenyl-chloroformate	100% (cytidine 2,3, cyclic phosphate)	50% to 70%	Effect of modification on the activity toward different substrate sizes	Veronese *et al.* (1985)
			65% to 50% (ribonucleic acid)	50% to 70%		
Sphingomyelinase	5000	Hydroxysuccinimide	70% (sphingomyelin), 0% (haemolysis)	100%	Effect on membrane-attacking activity	Matsuyama *et al.* (1993)
Superoxide dismutase	5000	Cyanuric chloride	100%	60–80%	Evaluation of cellular uptake of PEG–enzymes	Beckman *et al.* (1988)
Superoxide dismutase	2100	Glycol ester 4 fluoro-3-nitrobenzoic acid	100%	9 chains	Evaluation of new reagents chromophorically labelled	Ladd and Snow (1993)
	5210		73%	10 chains		
Superoxide dismutase	1900	Trichlorophenyl-chloroformate	90%, 70% and 70%	30%, 50% and 75%	Evaluation of clearance, immunogenicity, toward M_r PEG and % modification	Veronese *et al.* (1985)
Superoxide dismutase	5000	Cyanuric chloride	51%	19–20 PEG chains	Pharmacokinetics of modified enzymes in rats	Pyatak, Abuchowski and Davis (1980)
Streptokinase	2000, 5000	Carbonyldiimidazole			Pharmocokinetics and immunogenicity	Fears *et al.* (1992)
Uricase	5000	Cyanuric chloride	15 and 45% (uric acid)	43 and 37%	Immunogenicity	Nishimura *et al.* (1979), Nishimura, Matsushima and Inada (1981)
Urokinase	5000	Maleic anhydride			Effect of reversible polymer on activity	Garman and Kalindjian (1987)
Proteases for peptide synthesis						
Chymotrypsin	5000	Succinimidyl carbonate	65 and 29% (P–Gly–Gly–Leu–PheNAp)	63% and 79%	Effect of modification toward MW substrates	Chiu *et al.* (1993)
Papain	5000	Cyanuric chloride	87% (Benzoil-Arg-*p*-nitroaniline)	39%	Oligopeptide synthesis in benzene	Uemura *et al.* (1990)
Papain	5000	Cyanuric chloride	70% hydrolytic activity	37%	Solid-phase peptide synthesis in organic solvents	Sakurai *et al.* (1990)
Chymotrypsin	5000	Cyanuric chloride	68% hydrolytic activity	27%		
Thermolysin	5000	Cyanuric chloride	71% hydrolytic activity	27%		
Trypsin	5000	Cyanuric chloride	71% hydrolytic activity	23%		
Pepsin	5000	Cyanuric chloride	76% hydrolytic activity	20%		
Subtilisin Carlsberg	5000	Tresyl chloride	50% (*N*-tosyl-Arg-OMe)	n.d.	Aggregates of the PEG–enzyme in organic solvents	Khan *et al.* (1992)
Thermolysin	5000 chloroformate	Trichlorophenyl	>native (furylacryloyl-Gly-Leu-NH_2	20%	Peptide synthesis in organic solvents	Ferjancic, Puigserver and Gaertner (1988)
Trypsin	5000	Succinimidyl carbonate	110% to 92% (BAEE)	42 to 78% stability	Evaluation of a new reagent and its	Zalipsky, Seltzer and Menon-Rudolph (1992)

n.d., Not determined.

Behaviour of enzyme–PEG adducts

As a direct consequence of PEG modification of the surface of an enzyme, the enzyme is more resistant to various denaturing conditions (pH, elevated temperature) as was demonstrated with horseradish peroxidase (Fortier and Laliberté, 1993; Laliberté, Gayet and Fortier, 1994). The enzyme is also resistant to the action of proteases, a behaviour which is important for decreasing the immunogenicity of the protein. The plasma half-life of the enzyme is markedly increased. This will be seen below.

RESISTANCE TO PROTEASES

As was previously discussed for biomaterial covered with grafted PEG chains, repulsion of proteins to the surface results from the high segmental flexibility of the PEG that confers a strong mobility to the chain resulting in a high degree of steric exclusion (Lim and Herron, 1992). This explains in part why proteins, platelets and cells do not adhere on to PEG-treated materials (Kishida *et al.*, 1992; Amiji and Park, 1993; Llanos and Sefton, 1993). Similar reasons could explain why large substrates are not able to diffuse to the active site of the enzyme and why proteases cannot access the proteineous structure of a modified enzyme to hydrolyse peptide bonds. This proteolytic-resistant characteristic was demonstrated for several different proteins and enzymes (Kondo *et al.*, 1989; Sada *et al.*, 1991; Francis, Delgado and Fisher, 1992). The decrease in immunogenicity could be related to the lack of accessibility of the antigenic epitopes of the protein covered by PEG, which is a non-immunogenic polymer. It has been observed frequently that the protein–PEG adduct does not react with the antibody directed against the native protein (Abuchowski *et al.*, 1977; Sehon, 1992; Katre, 1993). In addition, without a complete Freund adjuvant, it is not possible to obtain antibody against PEG alone, except for those with very high molecular masses (Sehon, 1992). In the same manner, as the protein is more resistant to proteolysis, it is reasonable to think that when a modified protein is phagocytosed by macrophages, no elicitation of the humoral response occurs as there is no digestion of the modified protein. Thus, no antigen can be presented by the macrophage to the lymphocytes. There is no precise scientific explanation of why a modified protein cannot induce a humoral response, nevertheless the fact remains that the higher the number of PEG chains added at the surface, the lower are the possibilities of obtaining antibody. This phenomenon was observed for PEG of molecular mass higher than 1900.

BLOOD CLEARANCE

Modification of therapeutic proteins, enzymes or drugs with polyethylene glycol of various molecular masses offers several advantages, as mentioned in *Table 2*. One of these is related to the increase in serum residence time of the protein adducts when compared to the native proteins or enzymes. For example, *Table 5* shows the serum half-lives, in hours, of asparaginase and asparaginase–polyethylene glycol from *E. coli* in various species and as a function of the type of administration of the injection. In humans, the half-lives of asparaginase and asparaginase–PEG adducts are 7–8 hours and 384–600 hours, respectively, when injected intravenously (Park *et al.*,

1981). Numberous other examples can be found in recent review papers on the pharmacology and the pharmacokinetics of protein–PEG adducts (Francis, Delgado and Fisher, 1992; Zalipsky and Lee, 1992; Katre, 1993).

Table 5. Serum half-life (hours) of asparaginase or asparaginase–PEG adducts from *E. coli* following the administration of injection in various species

Species	Injection	ASNase	ASNase–PEG	References
Human	IM	30	144	Asselin *et al.* (1993)
	IM	33–43	–	Ho, Yap and Brown (1981)
	IV	7–8	384–600	Park *et al.* (1981)
	IV	7–29	–	Ho, Yap and Brown (1981)
Rhesus monkey	IM	146	–	Berg *et al.* (1993)
Dog	IV	24	?	Wada *et al.* (1990)
Rabbit	?	20	144	Ho *et al.* (1988)
Rat	IP	5	>72	Wada *et al.* (1990)
Mouse	IP	5	91	Abuchowski *et al.* (1984)

ASNase, asparaginase; IM, intramuscular; IP, intraperitoneal; IV, intravenous.

The increase in the half-life of a protein is of clinical importance because the therapeutic protein having a high $T_{1/2}$ in blood will maintain a therapeutic dosage during a longer time. This will reduce both the amount of enzyme–PEG adducts to be injected and the frequency of injections. Consequently, the therapeutic index will be higher for a modified protein than for the native one. This will also influence the cost of the treatment. As the modification of the surface by addition of PEG increases markedly the molecular mass and the hydrodynamic volume of the protein, the glomerular filtration of this modified protein is reduced, based on a molecular mass cut-off for the glomerular filtration of the protein of about 70 kDa.

Experiments conducted with rIL-2 (recombinant interleukin-2) have shown that the PEG adducts contribute to a decrease in renal clearance (Katre, Knauf and Laird, 1987; Knauf *et al.*, 1988) without affecting its antitumoral activity (Balemans *et al.*, 1993). The reduction of the renal clearance will favour the elimination of the protein adduct by the endothelial reticulum or by endocytosis, but at a rate of disappearance that will follow a model of second-order decay, as was demonstrated for various protein adducts. Effectively, a rapid phase is observed and this is related to a rapid endocytosis of the protein adduct, followed by the saturation of this system. A slow disappearance of the protein adduct is then observed and is probably the result of unspecific absorption. It is also expected, following surface modification, that cellular clearance of some modified proteins will be reduced due to the fact that their glycosylated parts are masked by PEG, avoiding specific interactions with cellular components. As an example, modified lactoferrin, which is normally cleared by hepatocytes by interaction between its carbohydrate components and cellular receptors, shows a dramatic decrease in its rate of clearance in the presence of hepatocytes when compared to unmodified lactoferrin (Beauchamp *et al.*, 1983).

Current biomedical applications and future developments of soluble enzymes and their pegylated derivatives

This subject has been reviewed recently (Klein and Langer, 1986; Goldberg, 1992;

Smith *et al.*, 1993). The technology of pegylation is now widespread, with the aim of increasing the therapeutic index of enzymes by limiting their loss by rapid *in vivo* degradation, by kidney clearance, by humoral response or by cellular components. *Table 1* gives an overview of diseases associated with enzyme deficiencies that can be overcome with appropriate enzyme treatments. More than 200 enzyme deficiencies related to genetic dysfunction have been reported in humans. Research in enzyme replacement therapy and gene therapy is currently in progress throughout the world to find solutions to these diseases. This is not a simple undertaking and it will take a considerable time for the establishment of an efficient gene therapy for each of these deficiencies. With this in mind, enzymotherapy is more than just an intermediate solution while awaiting the time when a successful gene therapy will be obtained. For example, in adenosine deaminase deficiency, enzymotherapy is the only palliative treatment available to limit the permanent damage during the time delay before gene therapy becomes operational in the patient. Three case applications involving the clinical use of enzymes or modified enzymes will now be presented.

CASE STUDY 1: ADENOSINE DEAMINASE DEFICIENCY

One of the most striking examples of the complementarity of the two approaches (gene therapy and enzymotherapy) is given by adenosine deaminase (ADA) deficiency. This results in defects in purine nucleotide metabolism, leading to severe immunodeficiency diseases afflicting the function of B and T lymphocytes. This enzyme catabolizes the deamination of adenosine (Ado), 2'-deoxyadenosine (dAdo) and many other 6-aminopurine nucleosides (Cory, 1992). An accumulation of deoxyadenosine triphosphate (dATP) was observed in patients with severe immunodeficiency. The dATP is an inhibitor of ribonucleoside reductase and subsequently of cellular replication. Therefore, by reducing the amount of dAdo in the blood, the accumulation of dATP is limited and the adenosine deaminase-deficient lymphocytes will be protected. The intracellular level of these metabolites is in partial equilibrium with the plasma, as shown by erythrocytes. Red cell transfusion, as well as bone marrow transplantation, has been used to palliate the ADA deficiency, despite the high risk associated with these treatments. It has been proposed (Polmar *et al.*, 1976; Hershfield *et al.*, 1987; Hershfield, Chaffee and Sorensen, 1993) to use purified ADA injected into the circulation to decrease the plasma level of the dAdo. As discussed previously, the free enzyme in circulating blood is rapidly eliminated but in the present case, no humoral response can occur since the immune system is dysfunctional. Therefore, to attain therapeutic levels of ADA, to increase the duration of the circulating enzyme in blood and to reduce the cost of treatment, the enzyme was pegylated (*Tables 1* and *4*). In a recent pharmacokinetic study in 29 patients with adenosine deaminase deficiency (Hershfield, Chaffee and Sorensen, 1993), it was shown that the pegylated ADA had a half-life in blood ranging between 3 and 6 days, when injected intramuscularly. The therapeutic level was selected to be 2–5 times greater than the normal level in total blood (red cell) and was maintained at >20 μmol h^{-1} ml^{-1}. After 6–8 months of PEG–ADA treatment, antibodies directed against ADA were found, as previously shown (Chaffee *et al.*, 1992). These did not impair treatment in the majority of patients. There was no evidence of hypersensitivity reactions. An increase of the lymphocyte responses to mitogens and restablishment of

lymphocyte counts were observed in 80% of the patients. The level of dAdo decreased by 100-fold during the first 2 months of the treatment and the low level of dAdo was maintained throughout the entire study. This indicates that immune function has been restored and that the use of ADA is beneficial. The opportunistic infections were also resolved and did not recur. The patient receives a weekly injection for the rest of his or her life. Due to the high cost of this non-curative treatment, ADA deficiency is therefore a good candidate for gene therapy. Effectively, a form of gene therapy is under development and consists of exposing *in vitro* the Il-2 dependent T cells of the patient to an ADA cDNA-bearing retroviral vector. This requires that the T cells of the patient are sufficiently mature. Consequently, a PEG–ADA treatment has to be performed before and continued during the reinjection of the T cells, to ensure their normal development.

CASE STUDY 2: GAUCHER'S DISEASE

Gaucher's disease is an inherited disease of lipid catabolism resulting from an absence of glucocerebrosidase which leads to the deposition of glucocerebrosides in macrophages of the reticuloendothelial system (Brady *et al.*, 1966). It is the most common lysosomal storage disease. Numerous mutations at the acid glucosidase locus on chromosome 1q21 are responsible for the lack of glucocerebrosidase activity in macrophages (Pastores, Sibille and Grabowski, 1993). Enzyme replacement therapy during the course of this disease is effective in treating the haematologic, hepatic and splenic damage (Barton *et al.*, 1991a,b) and it is also useful for partially reconstituting the vertebral body height after 16 months of enzymotherapy, as indicated in a recent case report (Hill *et al.*, 1993). This success was primarily achieved by the use of glucocerebrosidase isolated from placenta which can selectively interact with cells bearing mannose receptors, especially the reticuloendothelial cells (Stahl *et al.*, 1978). The major disadvantage of this enzymotherapy is its cost. It was demonstrated that 1 year of therapy for a patient exceeds more than $US 200 000, depending on the protocol used, i.e. self-administration of low doses or infusion of high doses at the hospital (Barton *et al.*, 1991a,b; Beutler *et al.*, 1991; Pastores, Sibille and Grabowski, 1993; Zimran *et al.*, 1993). A major breakthrough for this treatment would be to produce the enzyme by genetic engineering and to obtain the right post-translational modifications in terms of glycosylation that would preserve the self-targeting of the native enzyme.

CASE STUDY 3: ENZYMES IN THE ARSENAL FOR CANCER TREATMENTS

An antitumoural effect of guinea-pig serum was first observed by Kidd in 1953. Broome (1961) later demonstrated that asparaginase (L-asparaginase amidohydrolase, EC 3.5.1.1.) was the active factor in this serum. Intensive studies were initiated to understand the antitumour action of asparaginase and the role of depletion of asparagine on the proliferation of lymphoblastic leukaemia cells. At the same time, asparaginase was tested against a variety of solid tumours but disappointing results were obtained.

Following these findings, the therapy of neoplasia based on deprivation of essential amino acids became an attractive approach. However, it was shown subsequently that

dietary restriction of essential amino acids for control of tumour growth was inappropriate and unsuccessful (Roberts, 1981). Effectively, Marquez *et al.* (1989) and Medina, Marquez and Nunez de Castro (1992) established the dynamics of amino acid exchange between normal and neoplastic cells in mice. Three amino acids – glutamine, asparagine and serine – which are non-essential for normal cell growth, were found to be essential for the growth of tumour cells. In another study (Chuang, Yu and Wang, 1990), 17 amino acid-degrading enzymes were incubated individually with lymphocytes. The proliferation of lymphocytes was abolished only in the presence of lysine decarboxylase or asparaginase, and limited proliferations of 78% and 57%, compared to the control, were observed in the presence of arginase and tyrosinase, respectively.

Today, the use of asparaginase for the treatment of acute lymphoblastic leukaemia (ALL) and acute myeloblastic leukemia (AML) in children and adults, is one of the most widely studied and successful enzymotherapeutic applications. As the treatment of ALL or AML by asparaginase alone is ineffective, various antineoplasic drugs, such as vincristine, methotrexate, doxorubicin and VP16, are given together with the enzyme in different schedules and treatment regimens.

One of the first reviews (Capizzi and Cheng, 1981) on the use of asparaginase as a therapeutic agent against ALL discussed the toxicity and immunogenicity of asparaginase, the clinical importance of the inhibition of protein synthesis by asparagine depletion and, also, the low level of constitutive asparagine synthetase retrieved in normal cells. The explanation for tumour cell resistance to asparaginase treatment seems to be related to the fact that these cells can synthesize asparagine synthetase or that they have a high constitutive level of this enzyme. It appears difficult to evaluate the effectiveness of asparaginase treatment without evaluation of the level of constitutive asparagine synthetase in leukaemia cells. Martin *et al.* (1993) have shown by Western blot analysis of the protein content of murine leukaemia cells, both resistant (L5178Y D10/R) and non-resistant (L5178Y D10) to asparaginase treatment, that both cell types contain similar immunoreactive material with a molecular weight close to that asparagine synthetase. However, no enzymatic activity was detected in the sensitive cell preparation. It was hypothesized that sensitive cells were able to translate asparagine synthetase but with structural dysfunction. Further research was devoted to the understanding of the mechanism by which the enzyme asparaginase destroys leukaemia cells. Recently, Story *et al.* (1993) demonstrated experimentally in dogs that L-asparaginase kills lymphoma cells by apoptosis (Waring, Kos and Mullbacher, 1991), based on the analysis of cell morphology and DNA. They suggested that the enzyme is reducing or inhibiting protein synthesis to a level that prevents renewal of short-lived proteins which normally suppress activation of the mechanism of apoptosis (programmed cell death). It was also suggested that accumulation of aspartate induced a strong inhibition of the basic cellular energy mechanisms. Asparagine synthetase is responsible for the ATP-dependent synthesis of asparagine from glutamine and aspartic acid (Mehlhaff, Luehr and Schuuster, 1985).

To limit the side-effects of asparaginase treatment on normal cells, which have low levels of asparagine synthetase, and to initiate an early *de novo* synthesis of asparagine synthetase in these cells, it was proposed to deplete asparagine gradually to allow protein synthesis to continue normally. An experimental development has been presented recently based on *in vitro* procedures to evaluate the susceptibility of ALL

cells of patients to asparaginase (Asselin *et al.*, 1989) and to obtain precise quantification of asparagine levels in plasma (Asselin *et al.*, 1991). This should allow the evaluation of the level of asparagine synthetase and the time required to induce synthesis of the enzyme in normal cells. Such an analysis should be helpful for limiting the undesirable side-effects (hypoproteinaemia, decrease in coagulation rate, cytotoxicity and transient ischaemic cerebral lesion) (Priest *et al.*, 1982; Cetkosky *et al.*, 1993; Pihko *et al.*, 1993) of asparaginase treatment in normal cells.

An important point to note from recent clinical studies in acute lymphoblastic or myeloblastic leukaemia in adults or in children (Clavell *et al.*, 1986; Schiller *et al.*, 1993; Weiss *et al.*, 1993; Wells *et al.*, 1993) is that the enzyme L-asparaginase or the pegylated asparaginase are part of the arsenal of chemotherapeutic drugs that can be used in multidrug resistance regimens during the phases of induction and consolidation therapy. As an example, the Children's Cancer group, in a recent survey of 1294 patients treated in their programme, concluded that high doses of cytarabine and asparaginase during the intensification phase of treatment eliminated the necessity for prolonged maintenance therapy in childhood AML and improved the overall survival rate.

Hypersensitivity to asparaginase of *E. coli* has been observed to be as high as 50% of ALL patients (Capizzi *et al.*, 1971). Intramuscular administration of the enzyme led to lower allergic side-effects. Antibodies against asparaginase of *E. coli* do not cross-react with the asparaginase obtained from *Erwinia carotovora* or *E. chysanthemi*, the other main sources of the enzyme (Wade *et al.*, 1968). The *Erwinia* enzyme therefore offers an alternative to the *E. coli* enzyme when hyper-reactivity occurs in patients. To palliate the immunogenicity and the rapid blood clearance of asparaginase, several different approaches were attempted. These consisted of immobilization of the enzyme onto or into a solid matrix, such as polyacrylamide (Mori, Tosa and Chibata, 1974; Nadler and Updike, 1974), nylon (Mori, Tosa and Chibata, 1973; Chong and Chang, 1974). cellulose nitrate membrane (Chang, 1972/73) or dacron (Cooney, Weetall and Long, 1975). These implanted devices rapidly decreased the level of asparagine in blood. Due to a limited biocompatibility, dense collagen encapsulation of these devices occurred and the diffusion of asparagine into the device was then completely restricted. Recently, liposomes (Cruz *et al.*, 1993) or erythrocyte ghosts filled with asparaginase (Naql *et al.*, 1988; DeLoach *et al.*, 1990) were successfully evaluated. The use of a biocompatible hydrogel of PEG–BSA (D'Urso and Fortier, 1994a,b) loaded with asparaginase (D'Urso *et al.*, 1994) is currently being evaluated in our laboratory.

One of the successful approaches that has been approved for clinical use is based on the surface modification of the enzyme by covalent addition of polyethylene glycol (PEG). For this purpose, the surface was modified by the addition of PEG of molecular mass of 5000 to the free amino groups of the enzyme (Abuchowski *et al.*, 1984) and recently by using comb-shaped PEG-maleic acid (Kodera *et al.*, 1992) of high molecular mass. Depending on the percentage of free NH_2 groups of lysine modified and the nature of the activated PEG, various residual activities of asparaginase were obtained, ranging from 7% to 52%. The reader is referred to the review paper of Zalipsky and Lee (1992) for more details.

Limited clinical trials for the treatment of ALL have been performed using asparaginase–PEG adducts (Park *et al.*, 1981; Ho *et al.*, 1986; Yoshimoto *et al.*, 1986;

Jurgens *et al.*, 1988). A study with ALL in human patients (Asselin *et al.*, 1993) was recently published on the pharmacokinetics of asparaginase from *E. coli* and from *Erwinia* with pegylated asparaginase provided by Enzon Inc. (South Plainfield, NJ, USA). They found that neither the age, sex and risk category of the subject nor the dose and the number of intramuscular injections received (the first one or the thirtieth one) affected the serum half-life of the protein and the enzymatic activity of asparaginase. The results obtained with the pegylated asparaginase contrast with previously published results in humans and other species in that a relatively short half-life of pegylated asparagine was obtained (*Table 5*). For patients showing signs of hypersensitivity, only the pegylated asparaginase showed persistence in blood, with a $T_{1/2}$ of about 1.8 days. No conclusions can be drawn from this study concerning the mechanism by which asparaginase is eliminated from the circulation. The results suggest, however, that neither endoplasmic reticulum nor urinary excretion mechanisms were implicated. The blood clearance probably resulted from an endocytosis mechanism.

Similar approaches based on the selective deprivation of other amino acids in plasma have also been developed with the use of the enzyme asparaginase-glutaminase isolated from *Acinetobacter glutaminsificans* (Holcenberg *et al.*, 1979) and arginase (Savoca *et al.*, 1979, 1984). Glutamine is important for the growth of many types of tumours and these tissues may use this amino acid as an energy source. As a recent example, intraperitoneal injections of glutaminase on a daily basis into mice bearing an Ehrlich ascites tumour reduced the tumour size by 72% after 15 days and increased the life span by 46% (Pal and Maity, 1992). Glutaminase is cytotoxic against various types of asparaginase-resistant leukaemia cells and against some solid lymphoid human tumours. Relatively encouraging results (prolonged survival, although no remission) were obtained with patients having tumours refractory to conventional chemotherapy (Holcenberg *et al.*, 1979). In humans, the side-effects of glutaminase are similar to those observed during asparaginase treatments, although more pronounced in the former.

The depletion of arginine was also evaluated as an antineoplasic treatment, using arginase which hydrolyses arginine into urea and ornithine. It was demonstrated that the enzyme can inhibit the proliferation of normal and tumour cells (Currie, 1978). A few years ago, Savoca showed that the use of arginase–polyethylene glycol adducts in Ehrlich ascites tumours in mice was innocuous (Savoca *et al.*, 1979, 1984). There is much controversy concerning the use of arginase as a potent antitumoral drug. It is well known that the polyamine biosynthetic pathway is very active during the growth of various cancer cells. The plasma level of polyamines can be used as an indication of growth (Jiang, 1990) and depletion of polyamines leads to inhibition of tumour growth (Janne, Poso and Raina, 1978). With this concern in mind, various inhibitors of arginase and ornithine decarboxylase have been developed and used as antitumor agents (Trujillo-Ferrara *et al.*, 1992). This seriously contrasted with the use of arginase which can lead to an increase of polyamines (spermine, putrescine, spermidine) by increasing the ornithine content of the plasma.

Oxidative products of polyamine degradation, such as aldehydes, have been implicated in programmed cell death (Parchment and Pierce, 1989). New strategies for cancer treatment are currently under development using polyamine-degrading enzymes such as bovine serum amine oxidase which is responsible for the oxidative

deamination of polyamines leading to aldehydes, acrolein and hydrogen peroxide. These reaction products are highly toxic for cells (Averill-Bates *et al.*, 1993, 1994) and the toxicity is totally inhibited only in the presence of both catalase and aldehyde dehydrogenase. However, no pharmacokinetic studies of polyamine-degrading enzymes have been performed, and they are probably good candidates for derivatization by PEG, to increase their plasma half-life and to decrease their immunogenicity, for later use in humans.

Conclusion

There is no doubt that enzymotherapy is an emerging field that will provide a new class of biological drugs during the next decade. Enzymes should find wide applications as a replacement therapy in inherited genetic diseases, as scavengers to limit tissue damage during the course of inflammatory diseases and in various ischaemia/reperfusion injuries, as specific detoxification agents during acute chemical poisoning, as a temporary palliative agent during an acute organ failure and, also, as antineoplasic agents. Modification of enzymes, proteins, peptides and drugs by grafted polymers, especially polyethylene glycol, is currently the best strategy to solve problems related to the too-rapid clearance from blood and to the immunogenicity of the underivatized species. During the next decade, improvement of the purification technologies, lowering of the cost of technology and increased targeting of the enzyme–PEG adducts will be the main areas of research.

Acknowledgements

I would like to thank the Conseil de Recherches en Sciences Naturelles et en Génie and Université du Québec à Montréal for their financial support. I thank Dr D. Averill for her valuable comments and for the revision of the manuscript.

References

ABUCHOWSKI, A., VAN ES, T., PALCZUK, N.C. AND DAVIS, F.F. (1977). Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylne glycol. *Journal of Biological Chemistry* **252**, 3578–3581.

ABUCHOWSKI, A., KAZO, G.M., VERHOEST, C.R., JR, VAN ES, T., KAFKEWITZ, D., NUCCI, M.L., VIAU, A.T. AND DAVIS, F.F. (1984). Cancer therapy with chemically modified enzymes. I. Antitumor properties of polyethylene glycol–asparaginase conjugates. *Cancer Biochemistry Biophysics* **7**, 175–186.

AMIJI, M. AND PARK, K. (1993). Surface modification of polymeric biomaterials with poly(ethylene oxide), albumin, and heparin for reduced thrombogenicity. *Journal of Biomaterial Science – Polymer edition* **4**, 217–234.

ANDERSON, W.L. AND TOMASI, T.B. (1988). Polymer modification of antibody to eliminate immune complex and Fc binding. *Journal of Immunological Methods* **109**, 37–42.

ASSELIN, B.L., RYAN, D., FRANTZ, C.N., BERNAL, S.D., LEAVITT, P., SALLAN, S.E. AND COHEN, H.J. (1989). *In vitro* and *in vivo* killing of acute lymphoblastic leukemia cells by L-asparaginase. *Cancer Research* **49**, 4363–4368.

ASSELIN, B.L., LORENSON, M.Y., WHITIN, J.C., COPPOLA, D.J., KENDE, A.S., BLAKLEY, R.L. AND COHEN, H.J. (1991). Measurement of serum L-asparagine in the presence of L-asparaginase requires the presence of an L-asparaginase inhibitor. *Cancer Research* **51**, 6568–6573.

ASSELIN, B.L., WHITIN, J.C., COPPOLA, D.J., RUPP, I.P., SALLAN, S.E. AND COHEN, H.J. (1993). Comparative pharmacokinetic studies of three asparaginase preparations. *Journal of Clinical Oncology* **11**, 1780–1786.

AVERILL-BATES, D.A., AGOSTINELLI, E., PRZYBYTKOWSKI, E., MATEESCU, M.A. AND MONDOVI, B. (1993). Cytotoxicity and kinetic analysis of purified bovine serum amine oxidase in the presence of spermine in Chinese hamster ovary cells. *Archives of Biochemistry and Biophysics* **300**, 75–79.

AVERILL-BATES, D.A., AGOSTINELLI, E., PRZYBYTKOWSKI, E. AND MONDOVI, B. (1994). Aldehyde dehydrogenase and cytotoxicity of purified bovine serum amine oxidase and spermine in Chinese hamster ovary cells. *Biochemistry and Cellular Biology* **72**, 36–42.

BAILEY, F.E. AND KOLESKE, J.V. (1967). Configuration and hydrodynamic properties of the polyoxyethylene chain in solution. In *Nonionic Surfactants* (M.J. Schirk, Ed.), pp. 794–822. Marcel Dekker, New York.

BALEMANS, L.T.M., MATTIJSSEN, V., STEERENBERG, P.A., VAN DRIEL, B.E.M., DE MULDER, P.H.M. AND DEN OTTER, W. (1993). Locoregional therapy with polyethylene glycol-modified interleukin-2 of an intradermally growing hepatocellular carcinoma in the guinea pig induces T-cell-mediated antitumor activity. *Cancer Immunology Immunotherapy* **37**, 7–14.

BARTON, N.W., BRADY, R.O., DAMBROSIA, J.M., DI BISCEGLIE, A.M., DOPPELT, S.H., HILL, S.C., MANKIN, H.J., MURRAY, G.J., PARKER, R.I. AND ARGOFF, C.E. (1991a). Replacement therapy for inherited enzyme deficiency-macrophage-targeted glucocerebrosidase for Gaucher's disease. *New England Journal of Medicine* **324**, 1464–1470.

BARTON, N.W., BRADY, R.O., MURRAY, G.J., ARGOFF, C.E., GREWAL, R.P., KIAN-TI, Y., DAMBROSIA, J.M., ADRIAN, M., DI BISCEGLIE, A.M., HILL, S.C., PARKER, R.I., DOPPELT, S.H. AND MANKIN, H.J. (1991b). Enzyme-replacement therapy for Gaucher's disease. *New England Journal of Medicine* **325**, 1811.

BEAUCHAMP, C.O., DADDONA, P.E. AND MENAPACE, D.P. (1984). Properties of a novel PEG derivative of calf adenosine deaminase. *Advanced Experimental Medicine and Biology* **165A**, 47–52.

BEAUCHAMP, C.O., GONIAS, S.L., MENAPACE, D.P. AND PIZZO, S.V. (1983). A new procedure for the synthesis of polyethylene glycol–protein adducts; effects on function, receptor recognition and clearance of superoxide dismutase, lactoferrin, and α_2-macroglobulin. *Analytical Biochemistry* **131**, 25–33.

BECKMAN, J.S., MINOR, R.L., JR, WHITE, C.W., REPINE, J.E., ROSEN, G.M. AND FREEMAN, B.A. (1988). Superoxide dismutase and catalase conjugated to polyethylene glycol increases endothelial enzyme activity and oxidant resistance. *Journal of Biological Chemistry* **263**, 6884–6892.

BEECHER, J.E., ANDREWS, A.T. AND VULFSON, E.N. (1990). Glycosidase in organic solvents: II Transgalactosylation by polyethylene glycol-modified β-galactosidase. *Enzyme Microbiol Technology* **12**, 955–959.

BERG, S.L., BALIS, F.M., MCCULLY C.L., GODWIN, K.S. AND POPLACK, D.G. (1993). Pharmacokinetics of PEG–L-asparaginase and plasma and cerebrospinal fluid L-asparagine concentrations in the rhesus monkey. *Cancer Chemotherapy and Pharmacology* **32**, 310–314.

BEUTLER, W., KAY, A.C., SAVEN, A., GARVER, P., THURSTON, D.W. AND ROSENBLOOM, B.E. (1991). Enzyme-replacement therapy for Gaucher's disease. *New England Journal of Medicine* **325**, 1809–1810.

BODE, H.J. (1976). SDS-polyethyleneglycol electrophoresis, a possible alternative to SDS-polyacrylamide gel. *FEBS Letters* **65**, 56–58.

BOEHRINGER MANNHEIM (1988–1989). *Biochimica, Methods for Clinical Chemistry Research*, pp. 1–118.

BRADY, R.O., KANFER, J.N., BRADLEY, R.M. AND SHAPIRO, D. (1966). Demonstration of a deficiency of glucocerebroside-cleaving enzyme in Gaucher's disease. *Journal of Clinical Investigation* **45**, 1112–1115.

BROOME, J.D. (1961). Evidence that the L-asparaginase activity of guinea-pig serum is responsible for its antilymphoma effects. *Nature* **191**, 1114–1115.

CALICETI, P., MONFARDINI, C., SARTORE, L., SCHIAVON, O., BACCICHETTI, F., CARLASSARE, F. AND VERONESE, F.M. (1993). Preparation and properties of monomethoxy poly(ethylene glycol) Doxorubicin conjugates linked by an amino acid or a peptide as spacer. *Il Pharmaco* **48**, 919–932.

CAPIZZI, C.R.B. AND CHENG, Y.-Ć. (1981). Therapy of neoplasia with asparaginase. In *Enzymes as Drugs* (J.S. Holcenberg and J. Roberts, Eds), pp. 1–24. Wiley Interscience, New York.

CAPIZZI, C.R.B., SKEEL, J.R., CREASEY, W., ZANES, R., OLAYON, C., PETERSON, R. AND HANDSCHUMACHER, R. (1971). L-Asparaginase: clinical, biochemical, pharmacological and immunological studies. *Annals of International Medicine* **74**, 893–901.

CETKOVSKY, P., KOZA, V., CEPELAK, V., VIT, L. AND SIGUTOVA, P. (1993). Haemostatic changes in patients with acute lymphoblastic leukaemia during and after remission induction therapy. *Fibrinolysis* **7**, 386–390.

CHAFFEE, S., MARY, A., STIEHM, E.R., GIRAULT, D., FISCHER, A. AND HERSHFIELD, M.S. (1992). IgG antibody response to polyethylene glycol modified adenosine deaminase in patients with adenosine deaminase deficiency. *Journal of Clinical Investigation* **89**, 1643–1651.

CHANG, T.M.S. (1972/73). L-asparaginase immobilized within semipermeable microcapsules: *in vitro* and *in vivo* stability. *Enzyme* **14**, 95–104.

CHICZ, R.M. AND REGNIER, F.E. (1990). High performance liquid chromatography: effective protein purification by various chromatographic modes. *Methods in Enzymology* **182**, 393–421.

CHIU, H.-C., ZALIPSKY, S., KOPECKOVA, P. AND KOPECEK, J. (1993). Enzymatic activity of chymotrypsin and its poly(ethylene glycol) conjugates toward low and high molecular weight substrates. *Bioconjugate Chemistry* **4**, 290–295.

CHONG, E.D.S. AND CHANG, T.M.S. (1974). *In vivo* effects of intraperitoneally injected L-asparaginase solution and L-asparaginase immobilized within semipermeable nylon microcapsules with emphasis on blood L-asparaginase, 'Body' L-asparaginase, and plasma L-asparagine levels. *Enzyme* **18**, 218–239.

CHUANG, J.C., YU, C.L. AND WANG, S.R. (1990). Modulation of lymphocyte proliferation by enzymes that degrade amino acids. *Clinical and Experimental Immunology* **82**, 469–492.

CLAVELL, L.A., GELBER, R.D., COHEN, H.J. ET AL. (1986). Four agent induction and intensive asparaginase therapy for treatment of childhood acute lymphoblastic leukemia. *New England Journal of Medicine* **315**, 657–663.

COONEY, D.A., WEETALL, H.H. AND LONG, E. (1975). Biochemical and pharmacologic properties of L-asparaginase bounded to dacron vascular prostheses. *Biochemical Pharmacology* **24**, 503–515.

CORY, J.G. (1992). Purine and pyrimidine nucleotide metabolism. In *Textbook of Biochemistry with Clinical Correlations* (T.M. Devlin, Ed.) (3rd Edn), pp. 529–575. Wiley-Liss, New York.

CRUZ, M.E.M., GASPAR, M.M., LOPES, F., JORGE, J.S. AND PEREZ-SOLER, R. (1993). Liposomal L-asparaginase: *in vitro* evaluation. *International Journal of Pharmaceutics* **96**, 67–77.

CURRIE, G.A. (1978). Activated macrophages kill tumor cells by releasing arginase. *Nature* **273**, 758–759.

DAVIS, F.F., ABUCHOWSKI, A., VAN ES, T., PALCZUK, N.C., SACOVA, K., CHEN, R.H.-L. AND PYATAK, P. (1980). Soluble nonantigenic polyethylene glycol-bound enzymes. In *Biomedical Polymers* (E.P. Goldberg and A. Nakajima, Eds), pp. 441–451. Academic Press, New York.

DELGADO, C., PATEL, J.N., FRANCIS, G.E. AND FISHER, D. (1990). Coupling of poly(ethylene glycol) to albumin under very mild conditions by activation by tresyl chloride: characterization of the conjugate by partitioning in aqueous two-phase systems. *Biotechnology and Applied Biochemistry* **12**, 119–128.

DELLACHERIE, E. AND LÉONARD, M. (1991). Improvement of oxygen-carrying properties of human hemoglobin by chemical modification with a benzene hexacarboxylate-monosubstituted polyoxyethylene. *Journal of Protein Chemistry* **10**, 61–67.

DELOACH, J.R., ANDREWS, K., SATTERFIELD, W. AND KEELING, M. (1990). Intraperitoneal

administration of carrier erythrocytes in dogs: an improved method for delivery of L-asparaginase. *Biotechnology and Applied Biochemistry* **12**, 331–335.

D'URSO, E.M. AND FORTIER, G. (1994a). New hydrogel based on poly(ethylene glycol) cross-linked with bovine serum albumin. *Biotechnology Techniques* **8**, 71–76.

D'URSO, E.M. AND FORTIER, G. (1994b). New bioartificial polymeric material: poly(ethylene glycol) cross-linked with albumin. 1–Synthesis and swelling properties. *Journal of Bioactive and Compatible Polymers* **9**.

D'URSO, E.M., JEAN-FRANÇOIS, J., DOILLON, C.J. AND FORTIER, G. (1994). New family of hydrogels as matrix for enzyme immobilization: biomedical application. *Journal of Artificial Cells, Blood Substitutes and Immobilization Biotechnology* **22**, A14.

FEARS, R., HEARN, J., STANDRING, R., ANDERSON, J.L. AND MARDER, V.J. (1992). Lack of influence of pretreatment antistreptokinase antibody on efficacy in a multicenter patency comparison of intravenous streptokinase and anistreplase in acute myocardial infarction. *American Heart Journal* **124**, 305–314.

FERJANCIC, A., PUIGSERVER, A. AND GAERTNER, H. (1988). Unusual specificity of polyethylene glycol-modified thermolysin in peptide synthesis catalyzed in organic solvents. *Biotechnology Letters* **10**, 101–106.

FIELDS, R. (1971). The measurement of amino groups in proteins and peptides. *Biochemistry Journal* **124**, 581–590.

FLOHÉ, L. (1988). Superoxide dismutase for therapeutic use: clinical experience, dead ends and hopes. *Molecular and Cellular Biochemistry* **84**, 123–131.

FORTIER, G. AND LALIBERTÉ, M. (1993). Surface modification of horseradish peroxidase with poly(ethylene glycol) of various molecular masses. I. Preparation of the reagents and characterization of horseradish peroxidase-poly(ethylene glycol) adducts. *Biotechnology and Applied Biochemistry* **17**, 115–130.

FRANCIS, E.G., DELGADO, C. AND FISHER, D. (1992). PEG-modified proteins. In *Stability of Protein Pharmaceuticals, part B.* In Vivo *Pathways of Degradation and Strategies for Protein Stabilization* (T.J. Ahern and M.C. Manning, Eds), pp. 235–263. Plenum Press.

GARMAN, A.J. AND KALINDJIAN, S.B. (1987). The preparation and properties of novel reversible polymer–protein conjugates. 2-omega-methoxypolyethylene (5000) glycoxymethylene-3-methylmaleyl conjugates of plasminogen activator. *FEBS Letters* **223**, 361–365.

GIENFREDA, L. AND SCARFI, M.R. (1991). Enzyme stabilization: state of the art. *Molecular and Cellular Biochemistry* **100**, 97–128.

GOLDBERG, D.M. (1992). Enzymes as agents for the treatment of disease. *Clinica Chimica Acta* **206**, 45–76.

GREENWALD, R.A. (1990). Superoxide dismutase and catalase as therapeutic agents for human diseases. *Free Radical Biology and Medicine* **8**, 201–209.

GUILBAULT, G.G. (1984). *Analytical Uses of Immobilized Enzymes.* Marcel Dekker, New York.

GUPTA, M.N. (1991). Thermostabilization of proteins. *Biotechnology and Applied Biochemistry* **14**, 1–11.

HADLEY, K.B. AND SATO, P.H. (1989). Catalytic activity of administered gulonolactone oxidase polyethylene glycol conjugates. *Enzyme* **42**, 225–234.

HARRIS, J.M. (1985). Laboratory synthesis of polyethylene glycol derivatives. *JMS Review of Macromolecular Chemistry and Physics* **C25**, 325–373.

HARRIS, J.M., SEDAGHAT-HERATI, M.R., SATHER, P.J., BROOKS, D.E. AND FYLES, T.M. (1992). Synthesis of new poly(ethylene glycol) derivatives. In *Poly(ethylene glycol) Chemistry. Biotechnological and Biomedical applications* (M.J. Harris, Ed.), pp. 371–382. Plenum Press, New York.

HERSHFIELD, M.S., CHAFFEE, S. AND SORENSEN, R.U. (1993). Enzyme replacement therapy with polyethylene glycol–adenosine deaminase in adenosine deaminase deficiency: overview and case reports of three patients, including two now receiving gene therapy. *Pediatric Research* **33**, S42–S48.

HERSHFIELD, M.S., BUCKLEY, R.H., GREENBERG, M.L., MELTON, A.L., SCHIFF, R., HATEM, C., KURTZBERG, J., MARKERT, M.L., KOBAYASHI, R.H., KOBAYASHI, A.L. (1987). Treatment of adenosine deaminase deficiency with polyethylene glycol modified adenosine deaminase. *New England Journal of Medicine* **316**, 589–596.

HILL, S.C., PARKER, C.C., BRADY, R.O. AND BARTON, N.W. (1993). MRI of multiple plastyspondyly in Gaucher disease: response to enzyme replacement therapy. *Journal of Computer Assisted Tomography* **17**, 806–809.

HO, D.H., YAP, H.Y., BROWN, N. *et al.* (1981). Clinical pharmacology of intramuscularly administered L-asparaginase. *Journal of Clinical Pharmacology* **21**, 72–78.

HO, D.H., BROWN, N.S., YEN, A., HOLMES, R., KEATING, M., ABUCHOWSKI, A., NEWMAN, R.A. AND KRAKOFF, I.H. (1986). Clinical pharmacology of polyethylene glycol–L-asparaginase. *Drug Metabolism Disposition* **14**, 349–352.

HO, D.H., WANG, C.Y., LIN, J.R., BROWN, N., NEWMAN, R.A. AND KRAKOFF, I.H. (1988). Polyethylene glycol–L-asparaginase and L-asparaginase studies in rabbits. *Drug Metabolism Disposition* **16**, 27–29.

HOLCENBERG, J.S., BORELLA, L.D., CAMITTA, B.M. AND RING, B.J. (1979). Human pharmacology and toxicology of succinylated *Acinetobacter* glutaminase-asparaginase. *Cancer Research* **39**, 3145–3151.

HOVI, L., ERA, P., RAUTONEN, J. AND SIIMES, M.A. (1993). Impaired muscle strength in female adolescents and young adults surviving leukemia in childhood. *Cancer* **72**, 276–281.

INADA, Y., NISHIMURA, H., TAKAHASHI, K., YOSHIMOTO, T., SAHA, R.A. AND SAITO, Y. (1984). Ester synthesis catalyzed by polyethylene glycol-modified lipase in benzene. *Biochemical and Biophysical Research Communications* **122**, 845–850.

JACKSON, C.-J.C., CHARLTON, J.L., KUZMINSKI, K., LANG, G.M. AND SEHON, A.H. (1987). Synthesis, isolation, and characterization of conjugates of ovalbumin with monomethoxypolyethylene glycol using cyanuric chloride as the coupling agent. *Analytical Biochemistry* **165**, 114–127.

JANECEK, S. (1993). Strategies for obtaining stable enzymes. *Process Biochemistry* **28**, 435–445.

JANNE, J., POSO, H. AND RAINA, A. (1978). Polyamines in rapid growth and cancer. *Biochimica et Biophysica Acta* **473**, 241–293.

JIANG, X. (1990). Determination of polyamines in urine of normal human and cancer patients by capillary gas chromatography. *Biomedical Chromatography* **4**, 73–77.

JURGENS, H., SCHWAMBORN, D., KORHOLZ, D., WAHN, V. AND GOBEL, U. (1988). Clinical experiments with polyethylene glycol bound *E. coli* L-asparaginase in patients with multiple recurrences of acute lymphoblastic leukemia. *Klinische Paediatrie* **200**, 184–189.

KATRE, N.V. (1993). The conjugation of proteins with polyethylene glycol and other polymers. Altering properties of proteins to enhance their therapeutic potential. *Advanced Drug Delivery Reviews* **10**, 91–114.

KATRE, N.V., KNAUF, M.J. AND LAIRD, W.J. (1987). Chemical modification of recombinant interleukin 2 by polyethylene glycol increases its potency in the murine Meth A sarcoma model. *Proceedings of the National Academy of Science, USA* **84**, 1487–1491.

KHAN, S.A., HALLING, P.J., BOSLEY, J.A., CLARK, A.H., PEILOW, A.D., PELAN, E.G. AND ROWLANDS, D.W. (1992). Polyethylene glycol-modified subtilisin forms microparticulate suspensions in organic solvents. *Enzyme and Microbial Technology* **14**, 96–100.

KIKKAWA, S., TAKAHASHI, K., KATADA, T. AND INADA, Y. (1989). Esterification of chiral secondary alcohols with fatty acid in organic solvents by polyethylene glycol-modified lipase. *Biochemistry International* **19**, 1125–1131.

KISHIDA, A., MISHIMA, K., CORRETGE, E., KONISHI, H. AND IKADA, Y. (1992). Interactions of poly(ethylene glycol)-grafted cellulose membranes with proteins and platelets. *Biomaterials* **13**, 113–118.

KLAUSNER, A. (1983). New applications emerging: scientists working on enzymes for therapy. *Biotechnology* **1**, 734–735.

KLEIN, M.D. AND LANGER, R. (1986). Immobilized enzymes in clinical medicine: an emerging approach to new drug therapies. *Trends in Biotechnology* **4**, 179–186.

KNAUF, M.J., BELL, D.P., HIRTZER, P., LUO, Z.P., YOUG, J.D. AND KATRE, N.V. (1988). Relationship of effective molecular size to systemic clearance in rats of recombinant interleukin-2 chemically modified with water soluble polymers. *Journal of Biological Chemistry* **263**, 15064–15070.

KNÜSLI, C., DELGADO, C., MALIK, F., DOMINE, M., TEJEDOR, M.C., IRVINE, A.E., FISHER, D.

AND FRANCIS, G.E. (1992). Polyethylene glycol (PEG) modification of granulocyte–macrophage colony stimulating factor (GM-CSF) enhances neutrophil priming activity but not colony stimulating activity. *British Journal of Haematology* **82**, 654–663.

KODERA, Y., TANAKA, H., MATSUSHIMA, A. AND INADA, Y. (1992). Chemical modification of L-asparaginase with a comb-shaped copolymer of polyethylene glycol derivative and maleic anhydride. *Biochemical and Biophysical Research Communications* **184**, 144–148.

KONDO, A., KISHIMURA, M., KATOH, S. AND SADA, E. (1989). Improvement of proteolytic resistance of immunoadsorbents by chemical modification with polyethylene glycol. *Biotechnology and Bioengineering* **34**, 532–540.

KUNITANI, M., DOLLINGER, G., JOHNSON, D. AND KRESIN, L. (1991). On line characterization of polyethylene glycol-modified proteins. *Journal of Chromatography* **588**, 125–137.

LADD, D.L. AND SNOW, R.A. (1993). Reagents for the preparation of chromophorically labeled polyethylene glycol–protein conjugates. *Analytical Biochemistry* **210**, 258–261.

LALIBERTÉ, M., GAYET, J.-C.H. AND FORTIER, G. (1994). Surface modification of horseradish peroxidase with poly(ethylene glycol) of various molecular masses. II Relation between the molecular masses of PEG and the stability of horseradish peroxidase–PEG aducts under various denaturing conditions. *Biotechnology and Applied Biochemistry* **20**.

LAZNICEK, M., SCHIAVON, O., CALICETI, P. AND VERONESE, F.M. (1993). Pharmacokinetics and distribution of ribonuclease and its monomethoxypoly(ethylene glycol) derivatives in rats. *Pharmacological Research* **28**, 153–161.

LEHMAN, J.D., DYKE, C., ABD-ELFATTAH, A., YEH, T., JR., DING, M., EZRIN, A. AND WECHSLER, A.S. (1992). Polyethylene glycol-conjugated superoxide dismutase attenuates reperfusion injury when administered twenty-four hours before ischemia. *The Journal of Thoracic and Cardiovascular Surgery* **6**, 1597–1601.

LENTSCH, S., AIMAR, P. AND OROZCO, J.L. (1993). Separation of albumin–PEG: Transmission of PEG through ultrafiltration membranes. *Biotechnology and Bioengineering* **41**, 1039–1047.

LEVY, Y., HERSHFIELD, M.S., FERNANDEZ- MEJIA, C., POLMAR, S.H., SCUDIERY, D., BERGEN, M. AND SORENSEN, R.V. (1988). Adenosine deaminase deficiency with late onset of recurrent infections. Response to treatment with polyethylene glycol-modified adenosine deaminase. *Journal of Pediatrics* **113**, 312–317.

LIM, K. AND HERRON, J.N. (1992). Molecular simulation of protein–PEG interaction. In *Poly(ethylene glycol) Chemistry. Biotechnological and Biomedical Applications* (M.J. Harris, Ed.), pp. 29–56. Plenum Press, New York.

LISI, P.L., VAN ES, T., ABUCHOWSKI, A., PALCZUK, N.C. AND DAVIS, F.F. (1982). Enzyme therapy I. Polyethylene glycol: β-glucuronidase conjugates as potential therapeutic agents in acid mucopolysaccaridosis. *Journal of Applied Biochemistry* **4**, 19–33.

LLANOS, G.R. AND SEFTON, M.V. (1993). Does polyethylene oxide possess a low thrombogenicity? *Journal of Biomaterial Science – Polymer Edition* **4**, 381–400.

LU, Y.-A. AND FELIX, A.M. (1993). Pegylated peptides I: solid-phase synthesis of Na-pegylated peptides using Fmoc strategy. *Peptide Research* **6**, 140–146.

MCGOFF, P., BAZIOTIS, A.C. AND MASKEWICZ, R. (1988). Analysis of polyethylene glycol modified superoxide dismutase by chromatographic, electrophoretic, light scattering, chemical and enzymatic methods. *Chemical Pharmacology Bulletin* **36**, 3079–3091.

MAEDA, H., SEYMOUR, L.W. AND MIYAMOTO, Y. (1992). Conjugates of anticancer agents and polymers: advantages of macromolecular therapeutics *in vivo*. *Bioconjugate Chemistry* **3**, 351–362.

MAEDA, H., KIMURA, M., SASAKI, I., HIROSE, Y. AND KONNO, T. (1992). Toxicity of bilirubin and detoxification by PEG–bilirubin oxidase conjugate. A new tactic for treatment of jaundice. In *Poly(ethylene glycol) Chemistry. Biotechnological and Biomedical Applications* (M.J. Harris, Ed.), pp. 153–169. Plenum Press, New York.

MARQUEZ, J., SANCHEZ-JIMÉNEZ, F., MEDINA, M.A., QUESADA, A.R. AND NUNEZ DE CASTRO, I. (1989). Nitrogen metabolism in tumor bearing mice. *Archives of Biochemistry and Biophysics* **268**, 667–675.

MARTIN, J.K., SUN, W., MORAGA-A, D., SCHUSTER, S.M. AND WYLIE, D.E. (1993). An

investigation into the mechanism of L-asparaginase resistance in L5178Y murine leukemia cells. *Amino Acids* **5**, 51–69.

MATSUYAMA, H., TOMITA, M., TAGUCHI, R. AND IKEZAWA, H. (1993). Alteration in reactivity of sphingomyelinase from *Streptomyces* sp. modified with a polyethylene glycol derivative. *Biological and Pharmaceutical Bulletin* **16**, 107–111.

MATTIASSON, B. AND ADLERCREUTZ, P. (1991). Tailoring the microenvironment of enzymes in water poor systems. *Trends in Biotechnology* **9**, 394–398.

MEDINA, M.A., MARQUEZ, J. AND NUNEZ DE CASTRO, I. (1992). Interchange of amino acids between tumor and host. *Biochemical Medicine and Metabolic Biology* **48**, 1–7.

MEHLHAFF, P.M., LUEHR, C.A. AND SCHUUSTER, S.M. (1985). Studies on the ammonia-dependent reaction of beef pancreatic asparagine synthetase. *Biochemistry* **24**, 1104–1110.

MORI, S., NAKATA, Y. AND ENDO, H. (1992). Enzymatic properties of polyethylene glycol-modified cholesterol esterase in organic solvents. *Biotechnology and Applied Biochemistry* **16**, 101–105.

MORI, T., TOSA, T. AND CHIBATA, I. (1973). Enzymatic properties of microcapsules containing asparaginase. *Biochimica et Biophysica Acta* **321**, 653–661.

MORI, T., TOSA, T. AND CHIBATA, I. (1974). Preparation and properties of asparaginase entrapped in the lattice of polyacrylamide. *Cancer Research* **34**, 3066–3068.

MOZHAEV, V.V. (1993). Mechanism based strategies for protein thermostabilization. *Trends in Biotechnology* **11**, 88–95.

MOZHAEV, V.V. AND MARTINEK, K. (1990). Structure stability relationships in proteins: a guide to approaches to stabilizing enzymes. *Advanced Drug Delivery Reviews* **4**, 387–419.

MUIZELAAR, J.P., MARMAROU, A., YOUNG, H.F., CHOL, S.C., WOLF, A., SCHNEIDER, R.L. AND KONTOS, H.A. (1993). Improving the outcome of severe head injury with the oxygen radical scavenger polyethylene glycol-conjugated superoxide dismutase: a phase II trial. *Journal of Neurosurgery* **78**, 375–382.

NADLER, H.L. AND UPDIKE, S.J. (1974). Gel entrapment of enzyme. *Enzyme* **18**, 150–160.

NAQL, A., DELOACH, J.R., ANDREWS, K., SATTERFIELD, W. AND KEELING, M. (1988). Determination of parameters for enzyme therapy using L-asparaginase entrapped in canine erythrocytes. *Biotechnology and Applied Biochemistry* **10**, 365–372.

NARUKAWA, H., MIYOSHI, S.-I. AND SHINODA, S. (1993). Chemical modification of *Vibrio vulnificus* metalloprotease with activated polyethylene glycol. *FEMS Microbiology Letters* **108**, 43–46.

NISHIMURA, H., MATSUSHIMA, A. AND INADA, Y. (1981). Improved modification of yeast uricase with polyethylene glycol, accompanied with nonimmunoreactivity towards anti-uricase serum and high enzymic activity. *Enzyme* **26**, 49–53.

NISHIMURA, H., ASHIHARA, Y., MATSUSHIMA, A. AND INADA, Y. (1979). Modification of yeast uricase with polyethylene glycol. Disappearance of binding ability towards anti-uricase serum. *Enzyme* **24**, 261–264.

NISHIMURA, H., TAKAHASHI, K., SAKURAI, K., FUJINUMA, Y., IMAMURA, Y., OOBA, M. AND INADA, Y. (1983). Modification of batroxobin with activated polyethylene glycol: reduction of binding ability towards anti-batroxobin antibody and retention of defibrinogenation activity in circulation of preimmunized dogs. *Life Sciences* **33**, 1467–1473.

OFOSU-APPIAH, W., RUGGIERO, C., DZIELAK, D.J. AND ANTZELEVITCH, C. (1993). The effects of PEG–interleukin-2 and interleukin-2 on essential hypertension and cellular immune function in the spontaneously hypertensive rat. *Clinical and Experimental Hypertension* **15**, 435–457.

PAL, S. AND MAITY, P. (1992). Investigation on glutamine amidohydrolase and glutamine aminotransferase activity in liver and plasma of EAC bearing mice following glutaminase therapy. *Cancer Letters* **66**, 225–231.

PARCHMENT, R.E. AND PIERCE, G.B. (1989). Polyamine oxidation, programmed cell death and regulation of melanoma in the murine embryonic limb. *Cancer Research* **49**, 6680–6686.

PARK, Y.K., ABUCHOWSKY, A., DAVIS, S. AND DAVIS, F. (1981). Pharmacology of *Escherichia coli*–asparaginase polyethylene glycol adduct. *Anticancer Research* **1**, 373–376.

PASTORES, G.M., SIBILLE, A.R. AND GRABOWSKI, G.A. (1993). Enzyme therapy in Gaucher

disease type I: dosage efficacy and adverse effects in 33 patients treated for 6 to 24 months. *Blood* **82**, 408–416.

PIHKO, H., TYNI, T., VIRKOLA, K., VALANNE, L., SAINIO, K., HOVI, L. AND SAARINEN, U.M. (1993). Transient ischemic cerebral lesions during induction chemotherapy for acute lymphoblastic leukemia. *Journal of Pediatrics* **123**, 718–724.

POLMAR, S.H., STERN, R.C., SCHWARTZ, A.L., WETZLER, E.M., CHASE, P.A. AND HIRSCHHORN, R. (1976). Enzyme replacement therapy for adenosine deaminase deficiency and severe combined immunodeficiency. *New England Journal of Medicine* **295**, 1337–1343.

PRIEST, J.R., RAMSEY, N.K.C., STEINHERZ, P.G. *et al.* (1982). A syndrome of thrombosis and hemorrhage complicating L-asparaginase therapy for childhood acute lymphoblastic leukemia. *Journal of Pediatrics* **100**, 984–989.

PYATAK, P.S., ABUCHOWSKI, A. AND DAVIS, F.F. (1980). Preparation of a polyethylene glycol: superoxide dismutase adduct, and an examination of its blood circulating life and anti-inflammatory activity. *Research Communications in Chemical Pathology and Pharmacology* **29**, 113–127.

ROBERTS, J. (1981). Therapy of neoplasia by deprivation of essential amino acids. In *Enzymes as Drugs* (J.S. Holcenberg and J. Roberts, Eds), pp. 63–76. Wiley Interscience, New York.

ROIG, M.G. AND KENNEDY, J.F. (1992). Perspectives for chemical modifications of enzymes. *Critical Reviews in Biotechnology* **12**, 391–412.

SADA, E., KATOH, S., YAMANAKA, K. AND ITAKURA, Y. (1991). Resistance to proteolysis of antibody ligands modified with polyethylene glycol. *Journal of Fermentation and Bioengineering* **71**, 137–139.

SAKURAI, K., KASHIMOTO, K., KODERA, Y. AND INADA, Y. (1990). Solid phase synthesis of peptides with polyetylene glycol-modified protease in organic solvents. *Biotechnology Letters* **12**, 685–688.

SARTORE, L., CALICETI, P., SCHIAVON, O. AND VERONESE, F.M. (1991a). Enzyme modification by mPEG with an amino acid or peptide as spacer arms. *Applied Biochemistry and Biotechnology* **27**, 55–63.

SARTORE, L., CALICETI, P., SCHIAVON, O., MONFARDINI, C. AND VERONESE, F.M. (1991b). Accurate evaluation method of the polymer content in monomethoxy (polyethylene glycol) modified proteins based on amino acid analysis. *Applied Biochemistry and Biotechnology* **31**, 213–222.

SAVOCA, K.V., ABUCHOWSKI, A., VAN ES, T., DAVIS, F.F. AND PALCZUK, N.C. (1979). Preparation of a non-immunogenic arginase by the covalent attachment of polyethylene glycol. *Biochimica et Biophysica Acta* **578**, 47–53.

SAVOCA, K.V., DAVIS, F.F., VAN ES, T., MCCOY, J.R. AND PALCZUK, N.C. (1984). Cancer therapy with chemically modified enzymes. II. The therapeutic effectiveness of arginase and arginase modified by covalent attachment of polyethylene glycol on the Taper liver tumor and the L5178Y murine leukemia. *Cancer Biochemistry Biophysics* **7**, 261–268.

SCHILLER, G., LEE, M., TERRITO, M., GAJEWSKI, J. AND NIMER, S. (1993). Phase II study of etoposide, ifosfamide, and mitoxantrone for the treatment of resistant adult acute lymphoblastic leukemia. *American Journal of Hematology* **43**, 195–199.

SCHWARTZ, M.K. (1992). Enzymes as prognostic markers and therapeuthic indicators in patients with cancer. *Clinica Chimica Acta* **206**, 77–82.

SEHON, A.H. (1992). Suppression of antibody responses by conjugates of antigens and monmethoxypoly(ethylene glycol) in *Poly(ethylene glycol) Chemistry. Biotechnological and Biomedical Applications* (M.J. Harris, Ed.), pp. 139–152. Plenum Press, New York.

SELISKO, B., DELGADO, C. AND FISHER, D. (1993). Analysis and purification of monomethoxypolyethylene glycol by vesicle and gel permeation chromatography. *Journal of Chromatography* **641**, 71–79.

SIROKMAN, G. AND FASMAN, G.D. (1993). Refolding and proton pumping activity of a polyethylene glycol-bacteriorhodopsin water-soluble conjugate. *Protein Science* **2**, 1161–1170.

SMITH, R.A.G., DEWDNEY, J.M., FEARS, R. AND POSTE, G. (1993). Chemical derivatization of therapeutic proteins. *Trends in Biotechnology* **11**, 397–403.

SNYDER, S.L. AND SOBOCINSKI, P.Z. (1975). An improved 2,4,6-trinitrobenzene sulfonic acid method for determination of amines. *Analytical Biochemistry* **64**, 284–288.

SOKOL, R.J., BUTLER-SIMON, N., CONNER, C., HEUBI, J.E., SINATRA, F.R., SUCHY, F.J., HEYMAN, M.B., PERRAULT, J., ROTHBAUM, R.J., LEVY, J., IANNACCONE, S.T., SHNEIDER, B.L., KOCH, T.K. AND NARKEWICZ, M.R. (1993). Multicenter trial of D-a-tocopheryl polyethylene glycol 1000 succinate for treatment of vitamin E deficiency in children with chronic cholestasis. *Gastroenterology* **104**, 1727–1735.

SOUPPE, J. AND URRUTIGOITY, M. (1989). Chemical modification of horseradish peroxidase and papain with w-diamino-poly(oxyethylene)/carboxymethyl bis(dithiocarboxylate) polycondensates. *New Journal of Chemistry* **13**, 503–506.

STAHL, P.D., RODMAN, J.S., MILLER, M.J. AND SCHLESINGER, P.H. (1978). Evidence for receptor-mediated binding of glycoproteins, glycoconjugates, and lysosomal glycosidases by alveolar macrophages. *Proceedings of the National Academy of Sciences of the USA* **75**, 1399–1403.

STOCKS, S.J., JONES, A.J.M., RAMVY, C.W. AND BROOKS, D. (1986). A fluorometric assay of the degree of modification of protein primary amines with polyethylene glycol. *Analytical Biochemistry* **54**, 232–234.

STORY, M.D., VOEHRINGER, D.W., STEPHENS, L.C. AND MEYN, R.E. (1993). L-asparaginase kills lymphoma cells by apoptosis. *Cancer Chemotherapy and Pharmacology* **32**, 129–133.

TAKAHASHI, K., AJIMA, A., YOSHIMOTO, T. AND INADA, Y. (1984a). Polyethylene glycol-modified catalase exibits unexpectedly high activity in benzene. *Biochemical and Biophysical Research Communications* **125**, 761–766.

TAKAHASHI, K., NISHIMURA, H., YOSHIMOTO, T., SAITO, Y. AND INADA, I. (1984b). A chemical modification to make horseradish peroxidase soluble and active in benzene. *Biochemical and Biophysical Research Communications* **121**, 261–265.

TAM, C.M. AND TREMBLAY, A.Y. (1991). Membrane pore characterization: comparison between single and multicomponent solute probe techniques. *Journal of Membrane Science* **57**, 271–287.

TANG, G., WHITE, J.E., GORDON, R.J., LUMB, P.D. AND TSAN, M.-F. (1992). Polyethylene glycol-conjugated superoxide dismutase protects rats against oxygen toxicity. *Journal of Applied Physiology* **74**(3), 1425–1431.

THIBEAULT, D.W., REZAIEKHALIGH, M. AND MABRY, S. (1993). Lung antioxidant enzymes and cardiopulmonary responses in young rats exposed to hyperoxia and treated intratracheally with PEG catalase and superoxide dismutase. *Experimental Lung Research* **19**, 137–151.

TRUJILLO-FERRARA, J., KOIZUMI, G., MUNOZ, O., JOSEPH-NATHAN, P. AND YANEZ, R. (1992). Antitumor effect and toxicity of two new active site directed irreversible ornithine decarboxylase and extrahepatic arginase inhibitors. *Cancer Letters* **67**, 193–197.

UEMURA, T., FUJIMORI, M., LEE, H.-H., IKEDA, S. AND ASO, K. (1990). Polyethylene glycol modified papain catalyzed oligopeptide synthesis from the esters of L-aspartic and L-glutamic acids in benzene. *Agricultural and Biological Chemistry* **54**, 2277–2281.

VERONESE, F.M., LARGAJOLLI, R., BOCCU, E., BENASSI, C.A. AND SCHIAVON, O. (1985). Surface modification of proteins. Activation of monomethoxy-polyethylene glycols by phenylchloroformates and modification of ribonuclease and superoxide dismutase. *Applied Biochemistry and Biotechnology* **11**, 141–152.

VESTLING, M.M., MURPHY, C.M., KELLER, D.A., FENSELAU, C., DEDINAS, J., LADD, D.L. AND OLSEN, M.A. (1993). A strategy for characterization of polyethylene glycol derivatized proteins. A mass spectrometric analysis of the attachment sites in polyethylene glycol-derivatized superoxide dismutase. *Drug Metabolism and Disposition* **21**, 911–917.

VISCO, C., BENASSI, C.A., VERONESE, F.M. AND MIGLIOLI, P.A. (1987). Purification, modification, physico-chemical and pharmacokinetic characterization of arginase, an enzyme of potential use in therapy. *Il Farmaco* **42**, 549–559.

WADA, H., IMAMURA, I., SAKO, M., KATAGIRI, S., TARUI, S., NISHIMURA, H. AND INADA, Y. (1990). Antitumor enzyme: polyethylene glycol-modified asparaginase. *Annals of the New York Academy of Sciences* **613**, 95–108.

WADE, H.E., ELSWORTH, R., HERBERT, D. *et al.* (1968). A new L-asparaginase with antitumor activity. *Lancet* **2**, 776–777.

WARING, P., KOS, F.J. AND MULLBACHER, A. (1991). Apoptosis or programmed cell death. *Medicine Research Review* **11**, 219–236.

WEISS, M., TELFORD, P., KEMPIN, S., KRITZ, A., SOGOLOFF, H., GEE, T., BERMAN, E., SCHEINBERG, D., LITTLE, C., GAYNOR, J., BELANGER, C., BERTINO, J., ABDREEFF, M., MCKENZIE, S. AND CLARKSON, B. (1993). Severe toxicity limits intensification of induction therapy for acute lymphoblastic leukemia. *Leukemia* **7**, 832–837.

WELLS, R.J., WOODS, W.G., LAMPKIN, B.C., NESBIT, M.E., LEE, J.W., BUCKLEY, J.D., VERSTEEG, C. AND HAMMOND, G.D. (1993). Impact of high-dose cytarabine and asparaginase intensification on childhood acute myeloid leukemia: a report from the Children's Cancer Group. *Journal of Clinical Oncology* **11**, 538–545.

WIEDER, K.J., PALCZUK, N.C., VAN ES, T. AND DAVIS, F.F. (1979). Some properties of polyethylene glycol: phenylalanine ammonia lyase adducts. *Journal of Biological Chemistry* **254**, 12579–12587.

WIRTH, P., SOUPPE, J., TRITSCH, D. AND BIELLMANN, J.-F. (1991). Chemical modification of horseradish peroxidase with ethanol-methoxypolyethylene glycol: solubility in organic solvents, activity and properties, *Bioorganic Chemistry* **19**, 133–142.

WOGHIREN, C., SHARMA, B. AND STEIN, S. (1993). Protected thiol-polyethylene glycol: a new activate polymer for reversible protein modification. *Bioconjugate Chemistry* **4**, 314–318.

YOSHIMOTO, T., NISHIMURA, H., SAITO, Y., SAKURAI, K., KAMISAKI, Y., WADA, H., SAKO, M., TSUJINO, G. AND INADA, Y. (1986). Characterization of polyethylene glycol-modified L-asparaginase from *Escherichia coli* and its application to therapy of leukemia. *Japanese Journal of Cancer Research* **77**, 1264–1270.

YOSHINGA, K. AND HARRIS, J.M. (1989). Effects of coupling chemistry on the activity of poly(ethylene glycol)-modified alkaline phosphatase. *Journal of Bioactive and Compatible Polymers* **4**, 17–24.

ZALIPSKY, S. AND LEE, C. (1992). Use of functionalized polyethylene glycols for modification of polypeptides. In *Poly(ethylene glycol) Chemistry. Biotechnological and Biomedical Applications* (M.J. Harris, Ed.), pp. 347–370. Plenum Press, New York.

ZALIPSKY, S., GILON, C. AND ZILKHA, A. (1983). Attachment of drugs to polyethylene glycols. *European Polymer Journal* **19**, 1177–1183.

ZALIPSKY, S., SELTZER, R. AND MENON-RUDOLPH, S. (1992). Evaluation of a new reagent for covalent attachment of polyethylene glycol to proteins. *Biotechnology and Applied Biochemistry* **15**, 100–114.

ZALIPSKY, S., SELTZER, R. AND NHO, K. (1991). Succinimidyl carbonates of polyethylene glycol. Useful reactive polymers for preparation of protein conjugates. In *Polymeric Drugs and Drug Delivery Systems* (R.L. Dunn and R.M. Ottenbrite, Eds), pp. 91–100. American Chemical Society Symposium Series No. 469, Washington, DC.

ZAWILSKA, K., ZOZULINSKA, M., TUROWIECKA, Z., BLAHUT, M., DROBNIK, L. AND VINAZZER, H. (1993). The effect of a long-acting recombinant hirudin (PEG–hirudin) on experimental disseminated intravascular coagulation (DIC) in rabbits. *Thrombosis Research* **69**, 315–320.

ZIMRAN, A., HOLLAK, C.E.M., ABRAHAMOV, A., VA OERS, M.H.J. KELLY, M. AND BEUTLER, E. home treatment with intravenous enzyme replacement therapy for Gaucher disease: an international collaborative study of 33 patients. *Blood* **82**, 1107–1109.