0273-2289/97/6701-02—0153$10.75

Chemical Modification of Acetylcholinesterase with Methoxypolyethylene Glycol

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Received November 13, 1996; Accepted February 20, 1997

ABSTRACT

Acetylcholinesterase (AChE) (EC 3.1.1.7) was modified with activated monomethoxypolyethylene glycol (mPEG). A decrease of 50% in the catalytic activity was measured during the coupling reaction and the change in the surface properties of AChE was used to separate by hydrophobic interaction chromatography the native and the modified enzyme. The native and the modified enzymes were found to have the same optimal-catalytic conditions. Moreover, the Michaelis constant of both enzymes were similar, whereas the V_m and the bimolecular-velocity constant calculated for organophosphorus inhibitors were slightly higher for the modified AChE. Finally, the modification with mPEG did not improve the thermal stability, whereas the stability in a few organic solvents increased.

Index Entries: Covalent modification; acetylcholinesterase; methoxypolyethylene glycol; stability.

Abbreviations: AChE, acetylcholinesterase; ASChl, acetylthiocholine iodide; BCA, Bicinchoninic acid; DTNB, 5,5′-dithiobis (2-nitrobenzoïc acid); ki, bimolecular velocity constant; $K_m$app, Apparent Michaelis constant; mPEG, methoxypolyethylene glycol; mPEGpn, methoxypolyethylene glycol pnitrophenyl carbonate; PE, parathion ethyl; Px, paraoxon ethyl; Vm, maximal velocity.

INTRODUCTION

Several authors prepare proteins conjugates with large molecular weight carbohydrates *(1,2)*, PEG *(3,4)* and other polymers *(5,6)* to improve

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their chemical and biochemical stability. PEGs were initially used to reduce immunogenicity and to increase blood clearance time for enzymotherapeutic applications *(7)*. They are also used to increase thermostability as described by Gaertner and Puigserver *(3)* for chymotrypsin or by Laliberté et al. *(8)* for horseradish peroxidase, and to study biocatalysts in organic solvents *(8)*. Their amphipathic properties make the PEGs suitable both to modify properties of proteins in water and to solubilize enzymes in organic solvents.

Chemical modification of lysine side chains with mPEG has been made in an attempt to enhance the thermal stability of AChE. The properties of the native and modified enzymes, as well as the kinetic parameters ($K_m$app, ki), were compared because covalent modification can change the specificity of the enzyme.

Elsewhere, in the laboratory, we are developing biosensors for the detection of organophosphorus insecticides *(9,10)* based on the inhibition of AChEs, as these pesticides have high mammalian toxicity *(11)*. Organophosphorus pesticides are slightly soluble in water and largely soluble in organic solvents, which are used for their extraction and concentration. With a view to carrying out the inhibition step of cholinesterase in organic solvents, mPEG-AChE stability is tested in various polar and apolar solvents.

In this work, the modification of AChE by mPEGpn was studied and the properties of the conjugate were investigated.

MATERIALS AND METHODS

AChE from electric eel (EC 3.1.1.7), ASChl, DTNB, mPEGpn (MW 5000), mPEG (MW 5000), and organic solvents (HPLC grade) were purchased from Sigma Co. (La Verpillere, France). The pesticides used: parathion ethyl [*O,O*-diethyl *O*-4-nitrophenyl phosphorothioate] and paraoxon ethyl [*O,O*-diethyl *O*-4-nitrophenyl phosphate] were obtained from CIL Cluzeau (France). All other reagents were of analytical grade.

Activity Measurement

The cholinesterase activity was determined by the method of Ellman et al. *(12)*. All the kinetic measurements were performed at 410 nm using a Hewlett-Packard spectrophotometer equipped with a thermostated cell. Initial velocities were recorded at 37°C in a 1-mL cell containing 0.3 m*M* DTNB and various concentrations of ASChl (0–2.5 m*M*) in 50 m*M* phosphate buffer pH 8.0. One unit is defined as the amount of AChE catalyzing the hydrolysis of 1 µmol of ASChl per min.

Protein Measurement

Proteins were then determined with the (BCA) protein reagent (Pierce) based on the reaction of proteins with Cu^{2+} in an alcaline medium.

Protein samples reacted with the Pierce reagent for one h at 60°C and their concentrations were determined by measuring the absorbance at 562 nm using bovine serum albumin (BSA) as protein standard.

Inhibition by Insecticides

The reaction of irreversible AChE inhibitors is pseudo-first order with respect to inhibitor concentration. Phosphorylation of the enzyme is preceded by the formation of a reversible-binding complex as follow:

$$E + PX \overset{K_d}{\rightleftharpoons} EPX \xrightarrow{k_2} EP + X$$
$$\xrightarrow{\qquad k_i \qquad}$$

where EPX is the reversible-binding complex, K_d the equilibrium constant and k_2 a phosphorylation-rate constant. The bimolecular-velocity constant k_i ($k_i = k_2/K_d$) is often used to determine the inhibition of an insecticide. Methods to determine these constants have been described elsewhere *(13)*.

AChE was inhibited by paraoxon and parathion and activity was measured at various times as mentioned above.

Covalent Attachment of mPEGpn to AChE

400 µg of AChE were added to 400 µL of 0.1 *M* borate buffer, pH 9.1 containing mPEGpn in order to obtain a final εNH_2-lysine groups/mPEGpn molar ratio 1:10. The reaction mixture was kept at 25°C under gentle stirring. Residual activities were measured at several times (0, 30, 60, 90, 120, 150, and 180 min). *p*-nitrophenol and mPEGpn in excess were removed by filtration on an ultrafiltration system (Amicon) equipped with membranes having a cutoff of 30,000. The retained material was concentrated using centrifugal filtration with ULTRAFREE-MC (Millipore), cutoff 10,000 in a 50 m*M* phosphate buffer, pH 8.0.

Purification of Modified AChE

The separation of the native and the modified enzymes were performed on an hydrophobic-interaction chromatography using a phenyl Sepharose column (TSK-gel phenyl 5PW, Tosohaas) eluted with a linear-decreasing gradient of ammonium sulfate (from 1–0 *M*) at a flow rate of 0.5 mL · min^{-1}. Fractions (0.5 mL) were collected and evaluated for cholinesterase activity using Ellman's reagent. Fractions containing the modified AChE were mixed and concentrated using ultrafiltration as previously described.

pH and Temperature Dependence

Enzymatic activities were measured adding 10 µL (0.1 U) of enzyme in 990 µL of 0.3 m*M* DTNB and 0.6 m*M* ASChl dissolved in 50 m*M* phos-

phate buffer varying from pH 6.0 to 9.0. The cell was thermostated at 30°C; 10 µL (0.1 U) of enzyme were added in a cell containing 990 µL of 0.3 m*M* DTNB and 0.6 m*M* ASChl dissolved in 50 m*M* phosphate buffer, pH 8.0, preheated at 25, 30, 37, 40, 50, and 60°C.

Enzyme Stability

Thermal inactivation was studied at several temperatures: 30°C, 40°C, and 50°C. Native and modified AChE were prepared in preheated tubes (0.8 U/mL) containing 50 m*M* phosphate buffer, pH 8.0. Samples (0.08 U) were withdrawn at indicated intervals to measure residual activity. Thermal inactivation of native AChE was also carried out in presence of free mPEG. AChE was prepared in preheated tubes containing mPEG: 8 mg/mL of 50 m*M* phosphate buffer, pH 8.0.

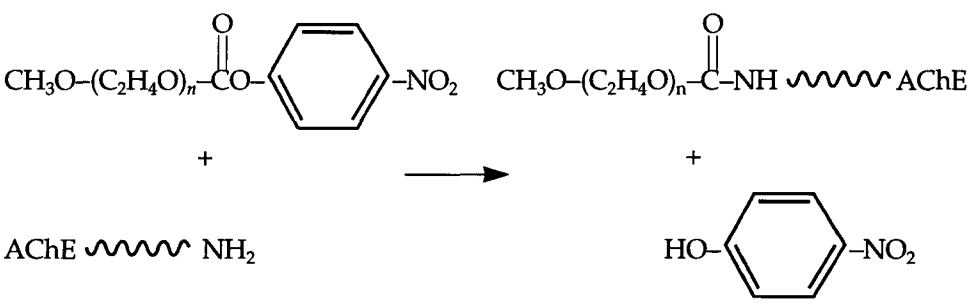
Inactivation in Organic Solvents

At room temperature 10 µL of a stock solution of AChE (100U/mL) prepared in 0.05 *M* phosphate buffer, pH 8.0, were incubated for 30 min with 300 µL of organic solvent. The enzyme was extracted with 3 mL of phosphate buffer and the Ellman colorimetric method performed using 100 µL of the aqueous phase when the solvent is nonmiscible and 110 µL when the solvent is water miscible.

RESULTS AND DISCUSSION

Covalent Linkage

AChE has been modified with a mPEG activated with p-nitrophenyl carbonate derivate, taking advantage of its clean reaction with primary amines in contrast with mPEG activated with cyanuric chloride *(14)*. The coupling reaction between the phenylcarbonate derivate and the protein amino groups occurs as follows *(15)*:



mPEG chains grafted onto the protein through stable urethane linkages.

Residual activity is measured during the modification reaction. Figure 1 compares the catalytic activity of native AChE, AChE with non-

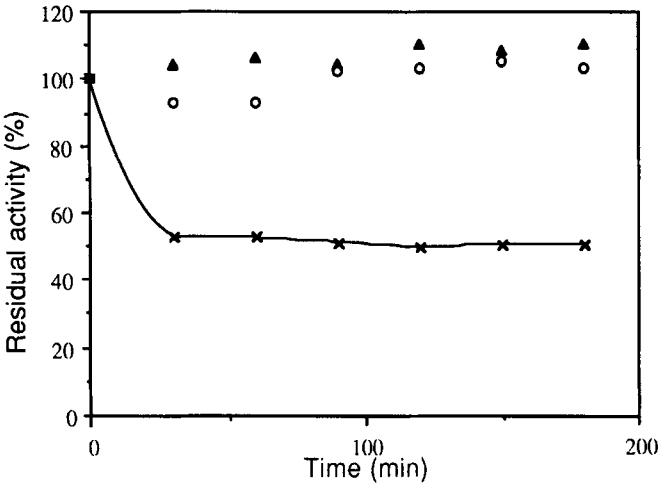


Fig. 1. Residual activity vs time during the coupling reaction: AChE with mPEGpn (×). Two standards are performed: AChE (▲) and AChE with mPEG (○). Aliquots of 0.1 U were withdrawn every 30 min.

activated mPEG, and AChE with mPEGpn in the conditions of the coupling reaction. The native enzyme and the enzyme incubated with nonactivated mPEG do not lose their catalytic activity, whereas the mPEGpn-AChE activity decreases. If, in some cases, upon attachment of PEG, some enzymes markedly enhance their hydrolytic rate *(9)*, most of them lose or only retain a small part of activity. Enzyme conformational changes or steric hindrances to the enzyme-substrate contact owing to the covalent linkage of the polymer to εNH_2-lysine groups of AChE can explain this phenomenon.

Purification

The chemical modification by mPEG increases molecular weight but also changes the surface characteristics of AChE. This surface modification can be used as a purification technique of the modified enzyme using hydrophobic interactions chromatography. Figure 2 shows the elution patterns obtained before and after the coupling reaction. Native and modified AChE are eluted at respectively 0.7 *M* and 0.4 *M* ammonium sulfate concentrations. The surface hydrophobicity thus increases with the amphipathic polymer. It considerably changes hydrophobicity by modifying εNH_2 polar groups. Moreover, the elution pattern shows that the coupling reaction is total; there is no more native AChE in the collected fractions.

Optimal Activity Conditions

Figure 3 compares the pH and temperature profiles of native and modified AChE. When temperature is over 40°C and pH reaches 9.0, the decomposition of DTNB is quite rapid and we face problems with blanks.

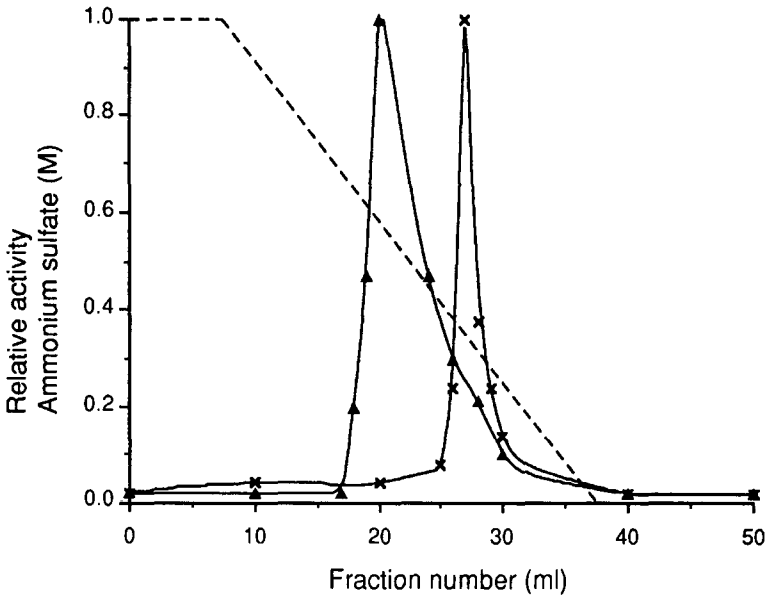


Fig. 2. Elution patterns on a hydrophobic interactions chromatography column of AChE before (▲) and after (×) the coupling reaction.

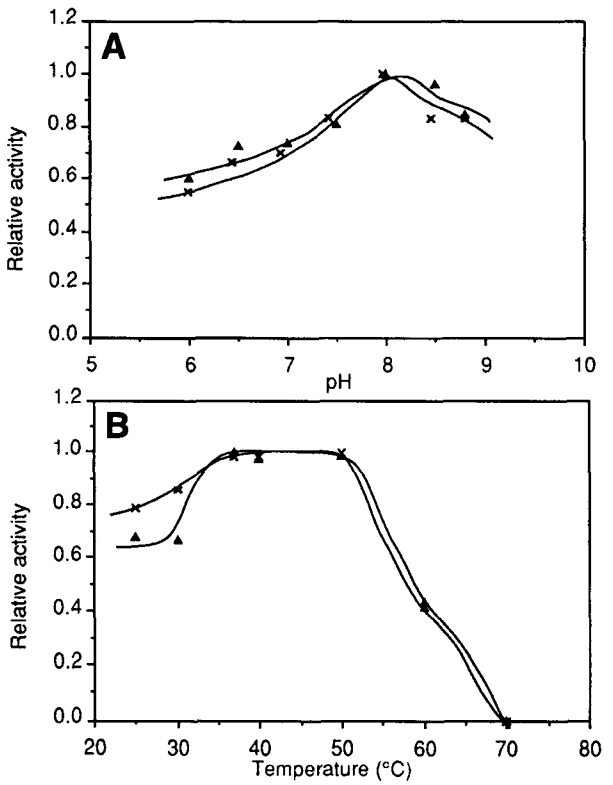


Fig. 3. **(A)** Effect of pH on ASChl hydrolysis by native (▲) and modified (×) AChE. **(B)** Effect of temperature on ASChl hydrolysis by native (▲) and modified (×) AChE.

Table 1
Kinetic Parameters of Substrate Catalysis (ASChl) and Kinetic Constants for the Inhibition of AChE and mPEG-AChE by Two Organophosphoruses

		AChE	mPEG-AChE
	K_m (μM)	60	65
	V_m (μmol.min^{-1})	0.045	0.075
	k_2 (min^{-1})	0.26	0.62
Paraoxon ethyl	K_D (M)	1.4×10^{-6}	1.4×10^{-6}
	k_i (M^{-1}.min^{-1})	1.9×10^{5}	4.4×10^{5}
	k_2 (min^{-1})	0.02	0.02
Parathion ethyl	K_D (M)	2.7×10^{-4}	2.1×10^{-4}
	k_i (M^{-1}.min^{-1})	74.0	95.0

pH 8.0 and 37°C are then used as optimal conditions of mPEG-AChE activity. As a consequence of the covalent linkage of a ligand to an enzyme, changes in the environment of the active site can result in changes of the optimum conditions for the catalytic activity. There is no change in the optimum pH and pH profile between the native and modified enzymes. The ionization of the amino-acid residues near the active site remains unaffected. It can be explained by the neutral nature of the polymer and the position of the lysine residues: far from the hydophobic active site. The optimal temperature and temperature profile measured for a short time (initial velocities) remain unchanged. The temperature stability was studied later by preincubating the enzyme.

Kinetic Parameters

Kinetic parameters ($K_m$app, k_i, V_m) using ASChl as substrate and PE and PX as inhibitors are reported in Table 1. They are not significantly affected by the modification even a low increase in V_m is reported for the conjugate. The K_m value of mPEG-AChE is similar to the native one, demonstrating that probably the affinity for the substrate is unaltered. No modification can occur near the active site (most of the lysine residues are on the opposite face); substrate can reach the catalytic triad of mPEG-AChE without steric hindrance. The attachment does not damage the active site or the accessibility of substrate and inhibitors to the active site. The bimolecular-velocity constant are slightly greater for the modified enzyme than the native one. The majority of modification methods do not significantly affect the kinetic constants as observed by other authors *(16,17)*.

We can noted that the PE inhibitor is a phosphorothioate that requires metabolic activation to the corresponding "oxon" form to be a potent

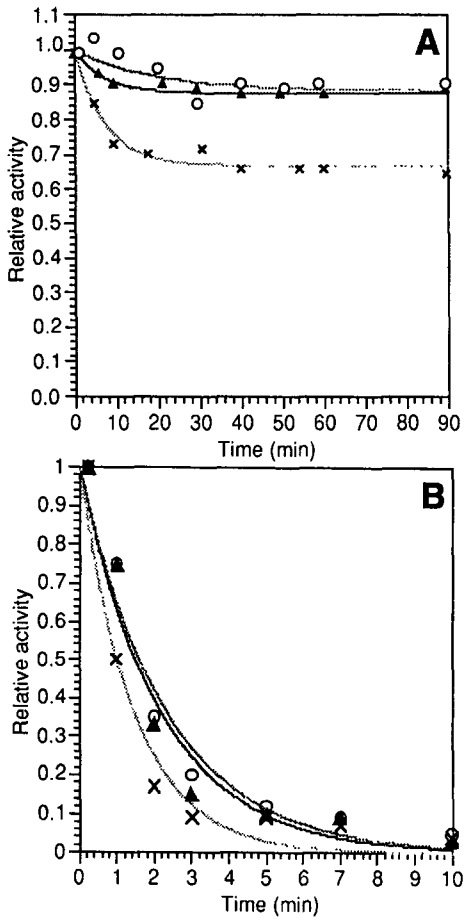


Fig. 4. Thermal stability of native (▲), modified (×), and native plus mPEG (○) AChE at 40°C **(A)** and 50°C **(B)**. Study was carried out in preheated tubes in a total volume of 250 µL. 10 µL of the sample were withdrawn to measure remaining activity in optimum conditions.

cholinesterase inhibitor. The k_i of PE (Table 1) is weak because of the use of their nonoxidized form. The inhibitor activity is compound-dependent and the oxon form reacts better, i.e., PX (oxon form of PE).

Thermal Inactivation

Figure 4A reports the denaturation measured at 40°C. AChE loses about 15% after 90 min and at 50°C (Fig. 4B) denaturation is complete within the first min. In both cases, the same behavior is observed using mPEG as an additive in contrast to Ye et al. *(18)*, who showed the stabilizing effect induced by PEG molecules on glucose oxidase. They noted the important role of the polymer length on enzyme stability with a maximal effect with 5 kDa PEG. But, according to them, the stabilizing effect of

additives is not an absolute effect and depends on the nature of the enzyme studied, its hydrophilic or hydrophobic character, and the degree of its interactions with the additive. Using AChE, free mPEG did not improve the stability of the native enzyme.

If we compare the modified and native enzyme with regard to temperature, mPEG-AChE loses twice as much activity as the native enzyme after 90 min at 40°C (Fig. 4A). At 50°C, there is no improvement of thermostability; both enzymes are totally denatured. Several authors *(3,8)* indicated an increased thermal stability after chemical modification of enzymes with PEG or other adducts *(19)*. The negative effect of the modification on the thermal stability could be compared to Ugarova et al. *(19)*. Based on circular-dichroism spectra of the modified peroxidase, these authors showed the effect of the modification on the conformational stability of the protein. Thus, the peroxidase was found to be more or less rigid and more or less thermostable depending on the number of modified lysines. The modification can make substantial alterations in the net charges of the proteins with a relatively small effect on stability up to a certain extent of modification where the stability of the proteins decreases sharply *(20)*. This indicates that the lysine residues involved in the modification are also involved in specific stabilizing interactions such as salt bridges in the protein.

Inactivation by Organic Solvents

Organophosphates and carbamates are slightly soluble in water and strongly soluble in organic solvents. In the laboratory, we have developed an amperometric biosensor *(21)* for the detection of these compounds in organic solvents based on a two-step method: inhibition of AChE in organic media and measurement of the remaining activity in a buffered medium. The stability of AChE has to be increased, especially in the polar solvents, which are detrimental for the enzymatic activity. The stability of AChE in many organic solvents has been correlated with log P (octanol-water partition coefficient) *(21)*.

As shown in Table 2, using the native enzyme, no detectable (acetonitrile and methanol) or low (dioxane and dichloromethane) activity was found when log $P < 2$. When log $P > 2$ (benzene, toluene, heptane), a remaining activity of respectively 50, 55, and 110%, compared to the phosphate buffer, was measured. The highest enzymatic activities were obtained in hydrophobic solvents as predicted by Laane et al. *(22)*.

Using the modified AChE, further observations are noticed:

1. the conjugate remains active (about 3%) after contact with acetonitrile or methanol,
2. the same behavior is observed when log $P > 2$ and an increased activity is measured with benzene (from 50 to 65%),

Table 2
Residual Activity After a 30 Min Contact with Various Solvents

Solvent	Log P	Residual activity (%)	
		Native AChE	Modified AChE
Dioxane	–1.1	2	60
Methanol	–0.76	0	3
Acetonitrile	–0.33	0	3
Dichloromethane	1.25	3	3
Benzene	2.0	50	65
Toluene	2.5	55	50
Heptane	4.0	110	105

3. the best result was found after contact dioxane: the remaining activity reaches 60% (2% for the native AChE), the reason of this remains unclear.

CONCLUSION

The coupling reaction of mPEG upon the lysine residues of AChE occurs in mild conditions: the reaction is complete and enzymatic activity is preserved. The optimal conditions (temperature and pH) are the same. Also for both enzymes, the kinetic parameters (affinity and rate constants) do not change either with the substrate or with competitive inhibitors. We also noticed that the modification decreases thermostability. Using the organic solvents has shown a low activity after contact with acetonitrile and methanol, whereas the native enzyme is completely denaturated, and an increased stability using dioxane. This covalent modification of acetylcholinesterase, then, can be advantageous to perform inhibition in organic medium. This aspect is currently under investigation.

REFERENCES

1. Fujita, T., Nishikawa, M., Tamaki, C., Takakura, Y., Hashida, M., and Sezaki, H. (1992), *J. Pharmacol. Exp. Ther.* **263, N°3,** 971–978.
2. Srivastava, R. A. (1991), *Enzyme Microb. Technol.* **13,** 164–170.
3. Gaertner, H. F. and Puigserver, A. (1992), *Enzyme Microb. Technol.* **14,** 150–155.
4. Inada, Y., Takahashi, K., Yoshimoto, T., Kodera, Y., Matsushima, A., and Saito, Y. (1988), *Tibtech.* **6,** 131–134.
5. Suh, J. and Hwang, B. K. (1992), *Bioorganic Chem.* **20,** 232–235.
6. Suh, J., Cho, Y., and Kwag, G. (1992), *Bioorganic Chem.* **20,** 236–244.
7. Narukawa, H., Miyoshi, S., and Shinoda S. (1993), *Microbiol. Lett.* **108,** 43–46.
8. Laliberté, M., Gayet, J-C., and Fortier, G. (1994), *Biotechnol. Appl. Biochem.* 397–413.
9. Marty, J.-L., Mionetto, N., and Rouillon, R. (1992), *Anal. Let.* **25,** 1389–1398.

10. Mionetto, N., Rouillon, R., and Marty, J.-L. (1992), *Z. Wasser-Abwasse-Forsch* **171,** 171–174.
11. Moretto, A. and Johnson, M. (1987), *Toxicology of Pesticides: Experimental, Clinical and Regulatory,* Springer-Verlag Berlin, H13, Heidelberg, Germany, pp. 33–48.
12. Ellman, J. C., Courtney, K. D., Andres, V., and Featherston, R. M. (1961), *Biochem. Pharmacol.* **7,** 88–95.
13. Main, A. R. (1979), *Pharmac. Ther.* **6,** 579–628.
14. Zalipsky, S. (1995), *Bioconjugate Chem.* **6,** 150–165.
15. Veronese, F. M., Largajolli, R., Boccù, E., Benassi, C. A., and Schiavon, O. (1985), *Appl. Biochem. Biotechnol.* **11,** 141–152.
16. Beecher, J. E., Andrews, A. T., and Vulfson, E. N. (1990), *Enzyme Microb. Technol.* **12,** 955–959.
17. Geiger, B., Von Specht, B. U., and Arnon, R. (1977), *Eur. J. Biochem.* **73,** 141–147.
18. Ye, W. N., Combes, D., and Monsan, P. (1988), *Enzyme Microb. Technol.* **10,** 498–502.
19. Ugarova, N. N., Rozhkova, G. D., and Berezin, I. V. (1979), *Biochimica Biophysica Acta* **570,** 31–42.
20. Kristjansson, M. M. and Kinsella, J. E. (1991), *Adv. Food Nutr. Res.* **35,** 237–316.
21. Mionetto, N., Marty, J-L., and Karube, I. (1994), *Biosens. Bioelectron.* **9,** 463–470.
22. Laane, C., Boeren, S., Hilhorst, R., and Veeger, C. (1986), in *Proc. of International Symposium,* Laane, C., Tramper, J., and Lilly, M. D. eds., Elsevier Science, Amsterdam, pp. 65–84.