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Surface Modification of Proteins

Activation of Monomethoxy-Polyethylene Glycols by Phenylchloroformates and Modification of Ribonuclease and Superoxide Dismutase

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ABSTRACT

A single-step method of activation of monomethoxy-polyethylene glycols suitable for its binding to polypeptides and proteins is proposed. Based on the reaction with 2,4,5-trichlorophenylchloroformate or *p*-nitrophenylchloroformate, it gives reactive PEG–phenylcarbonate derivatives. The PEG intermediate is stable on storage, the activating group is easily quantified, and the reaction with amino acid and proteins proceeds rapidly at pH near neutrality. The PEG derivatization of enzymes with this procedure is less inactivating than those previously reported. Ribonuclease and superoxide dismutase were modified and the effect of (a) bound polymer on clearance time in rats, (b) antibody recognition, and (c) on the enzymatic activity toward low and high molecular weight substrates were studied.

Index Entries: Monomethoxypolyethylene glycol activation; surface modification of proteins; superoxide dismutase modification by polymers; pharmacokinetics of polymer-modified proteins; proteins, surface modification of; polyethylene glycols, activation of

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monomethoxy-; phenylchloroformates, activation of monomethoxy-polyethylene glycols by; ribonuclease, modification by polymers.

INTRODUCTION

Derivatives of polyethyleneglycols (PEG) have proved to be useful in a variety of basic and applied biochemical fields such as protein modification, peptide synthesis, coenzyme and drug derivatization, polymer-binding reagents, surfactants, phase-transfer reagents, proteins, and cell purification. The activation of PEG is the most critical step in the utilization of the modified proteins, and this explains why a variety of chemical methods were thus far proposed.

Protein modification by PEG, monomethoxypolyethyleneglycol in particular, was recently proposed as a promising method for obtaining more suitable derivatives for medical applications. The PEG-bound proteins often present increased stability to proteolytic digestion, higher half-life times when injected into the bloodstream, and reduced immunological response (*1–5*). For this purpose, the hydroxy group of PEG has usually been activated by cyanuric chloride and then coupled to nucleophilic sites in proteins (*1*). The method, however, has several disadvantages since (a) the modification is unsuitable for enzymes possessing reactive—SH groups in the active site (6); (b) cyanuric chloride is toxic *per se* and its degradation products may also be toxic in vivo; (c) the modified protein may consist of species of higher molecular weight than expected on the basis of bound PEG, probably because of the third chloride of the coupling reagent that subsequently reacts, giving cross-linked products (*7*); and finally (d) the derivatives absorb in the UV region, impeding accurate spectroscopic studies.

As alternative procedures, succinimidylsuccinates of PEGs have been prepared (*8*); however, this procedure is limited by the easy hydrolysis of the ester between PEG and succinic acid. The carbinol function of PEG has also been converted to a carboxylic acid by a three- or a four-step procedure (*6, 9*) or by a two-step oxidation (*6*) followed by activation with hydroxysuccinimide and dicyclohexylcarbodiimide. These methods yielded activated PEGs that do not inactivate—SH dependent enzymes, but the purification of polymeric intermediates was difficult and time-consuming.

More recently, the polymer was reacted with carbonyldihymidazole to produce an activated PEG that binds the lysine amino groups of proteins (*10*). Apparently, a drawback of the described procedure lies in the difficulty in obtaining a highly activated polymer, leading to low yields of protein binding.

As a contribution to this theme, we are reporting here the activation of PEGs in a single-step procedure by 2,4,5-trichlorophenylchloroformate

or *p*-nitrophenylchloroformate to give active carbonates that react with amino residues of peptides or proteins to give stable carbamates. The procedure may be considered an extension to PEG chemistry of the recently proposed technique of activation of polysaccharidic resins by the same reagents, for amino ligand immobilization in affinity chromatography (*11*). The method was tested on ribonuclease A and superoxide dismutase (SOD); the enzyme activity of PEG-modified molecules has been tested against that of native enzyme. For superoxide dismutase, an enzyme used as an antiinflammatory drug, the influence of the extent of modification by PEG on the clearance time in rats and on the precipitation by anti-SOD antibodies was also investigated.

MATERIALS AND METHODS

Materials

PEG and cyanuric chloride were from the Aldrich Chemical Co. *p*-Nitro- and 2,4,5-trichlorophenylchloroformate were from Janssen. Ribonuclease A (RNase) from bovine pancreas, yeast ribonucleic acid, trinitrobenzene sulfonic acid, and xanthine oxidase were from Sigma. Cytidine 2',3'-cyclic phosphate from Ega Chemie; Bio Gel-A, 0.5 m was from Rio-Rad. Salts were reagent-grade from Merck and solvents from Baker. Cupro–zinc superoxide dismutase (SOD) from bovine erythrocytes was prepared in our laboratory using a slightly modified version of the method described by McCord and Fridovich (*12*). α-Z-Lys was prepared in our laboratory, and serum anti-SOD was kindly supplied by Prof. Neri of Instituto Sieroterapico Vaccinogeno Toscano (Sclavo, Siena). Rats were from Charles River, Como, Italy.

Enzymatic Assay

Assays of SOD were carried out according to the published method of McCord and Fridovich (*12*). RNase activity was measured by the increase in absorbance at 287 nm of a cytidine 2',3'-cyclic phosphate solution in Tris-acetate 0.1*M*, pH 7.6, buffer or by the decrease in absorbance at 300 nm using ribonucleic acid.

Protein, PEG, and —NH_2 Group Titration

The concentrations of unmodified proteins were calculated using the following extinction coefficients and molecular weight determinations

$$\text{SOD, } A^{1\%}_{1\text{cm},258\text{ nm}} = 3.31 \qquad m_r = 31{,}200 \text{ (12)}$$
$$\text{RNase, } A^{1\%}_{1\text{cm},280\text{ nm}} = 6.95 \qquad m_r = 13{,}700$$

The concentration of PEG-modified protein was estimated by amino acid analysis. The PEG concentration was estimated by the iodometric

method of Sims and Shaper (*13*), and the content of amino groups after peptide or protein modification was carried out using the trinitrobenzene sulfonate method (*14*).

Activation of PEG

To a solution of 1 g of PEG-1900 in 10 mL of dry pyridine, a 2.5*M* excess of 2,3,5-trichlorophenylchloroformate was added in small portions. After standing at room temperature for 24 h, 100 mL of dry ether was added. The precipitate was collected, washed with ether, redissolved in acetonitrile, and ethyl ether was added to faint turbidity, and the product was allowed to crystallize at 4°C. The product was obtained in 72% yield. *Anal.* calc. for $C_{94}H_{178}Cl_3$: C, 52.50; H, 8.34; Cl, 4.94. Found: C, 53.04; H, 8.25; Cl, 5.02. The same procedure was used to prepare activated PEG-5000, but lower volumes of ether were used for the precipitation from pyridine. The recrystallized product from acetonitrile–ether gave the following analysis: calc. for $C_{235}H_{461}O_{116}Cl_3$: Cl, 2.02. Found: Cl, 2.00.

A good alternative method for the activiated-PEG purification was to dissolve the polymer in warm ethanol (35–40°C) followed by crystallization at 0°C.

The PEG–*p*-nitrophenylcarbonate was obtained as follows: to a solution of 0.6 g of *p*-nitrophenylchloroformate in 50 mL of acetonitrile, 5 g of PEG-1900 and 0.29 g of triethylamine were added. After stirring for 24 h, the precipitated triethylammonium chloride was filtered, about 500 mL of ethyl ether were added and the solution was left to crystallize overnight at 4°C. The product was filtered, washed with ether, and recrystallized from acetonitrile–ether with an 80% yield. *Anal.* calc. for $C_{94}H_{18}O_{48}N$: N, 0.67. Found: N, 0.66.

The products were also analyzed by titration of the released 2,4,5-trichloro- or p-nitrophenol, after standing in 0.2*N* NaOH solution (*11*).

Coupling to Activated Amino Derivatives and Proteins

Activated PEG-1900 or 5000 was added to a solution of the protein or amino acid in phosphate or borate buffer, and the desired pH was maintained with a pH-stat while the amino groups were titrated by the trinitrobenzenesulfonate reaction.

Pharmacokinetic Assays

Plasma clearance evaluation was performed in Levis male rats (200–250 g) provided with food and water *ad libitum*. They were divided into groups of five and injected with 5 mg/kg of SOD or equimolar amount of PEG–SOD samples into the saphenous vein. The blood was collected in heparinized tubes from the tail vein on a time schedule vary-

ing according to the SOD species; after centrifugation it was assayed for enzymatic activity.

RESULTS

Properties of PEG Activated by Phenylchloroformates

Figure 1 shows the schematic representation of the reaction of PEG with phenylchloroformates to give phenylcarbonates, which in turn may react with unprotonated amines to give urethane derivatives. The intermediate phenylcarbonates are unstable at alkaline pH. In Fig. 2, the rate of the hydrolysis of the PEG 2,4,5-trichlorophenylcarbonate evaluated on the basis of the absorption of the released chlorophenol is reported. The hydrolysis follows first-order kinetics with a $T_{1/2}$ of 540, 270, and 70 min for the PEG-2,4,5-trichlorophenyl, $T_{1/2}$ of 300, 90, and 40 min for the PEG–*p*-nitrophenyl, respectively, at pH 8.3, 9, and 9.3. The absorbance spectrum of the released phenol in alkali represents an easy method to verify the extent of PEG activation. The activated PEG could be stored for at least 1 yr in the dry state without appreciable loss of activity.

Amino Acid and Protein Modification by PEG–Phenylcarbonates

In Fig. 3, the time course of reaction of activated PEG with α-Z-lysine is reported as a function of pH. As expected for a nucleophilic displacement, the reaction rate increases with the deprotonation of the amino group, the $T_{1/2}$ being 30, 18, and 6 min, respectively, at pH 8.3, 8.8, and 9.3 for the 2,4,5-trichlorophenyl derivative.

The modification of ribonuclease A by the same reagent in a molar ratio of 5 to 1 over the available amino groups is given in Fig. 4; the rate is also pH-dependent, but in this case a slowing down of the reaction with time is observed. This result may be explained only partially by the si-

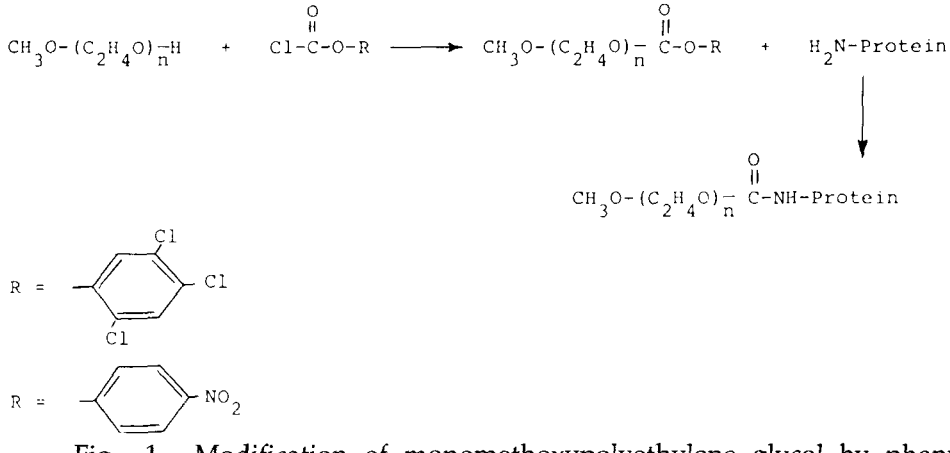

Fig. 1. Modification of monomethoxypolyethylene glycol by phenylchloroformate and reaction of the phenylcarbonate derivative with proteins or peptides amino groups.

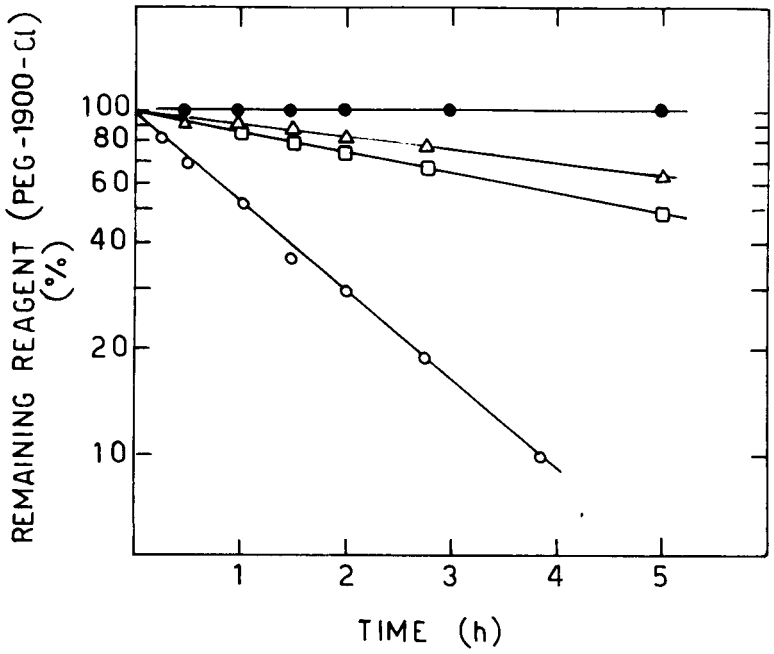

Fig. 2. Rate of hydrolysis of PEG 1900-ω-trichlorophenylcarbonate (1 mg/mL) in phosphate buffer, 0.1*M*, pH 7 (•); borate buffer, pH 8.5 (Δ), pH 9 (□), and pH 9.5 (○). The rate was assessed on the basis of the amount of trichlorophenol released as measured by UV absorption and by automatic titration with a pH-stat.

multaneous hydrolysis of the reagent added in a low molar excess. More probably it reflects different accessibilities for the amino groups of the protein molecule *per se*, or it is because of the hindrance of the previously bound PEG molecules. The modification is not accompanied by loss of enzyme activity as assessed by cytidine-2′,3′-cyclic phosphate, whereas a very large loss is detected against the macromolecular substrate ribonucleic acid.

It is worth reporting that when the PEG is bound to RNase by cyanuric chloride activation, complete loss of enzymatic activity is also observed towards cytidine-2′,3′-cyclic phosphate.

Modification of superoxide dismutase at pH 8.8 is reported in Fig. 5. The activity appears reduced to about 80% when 10 PEG molecules are bound to the protein and this is maintained while the modification proceeds.

The modification was also carried out on a preparative scale. In order to obtain SOD with an average of 3, 7, 14, or 17 molecules of bound PEG per enzyme molecule, the reaction was stopped by the addition of excess Gly-Gly after 10 and 30 min, and after 2.5 and 6 h, respectively. The separate samples were chromatographed on a Bio-gel column. The pattern of elution of the sample with an average of 14 mol of bound polymer is reported in Fig. 6. The fractions were analyzed by UV absorption to follow

both the protein elution and the released trichlorophenol by enzymatic activity and iodide titration for PEG concentration. The separation from the side products was also carried out by ultrafiltration using Amicon pH 10 membranes; but this method was less efficient. The colorimetric method of Sims and Shape (*13*) for PEG appeared to be very reliable and convenient.

The stability of the carbonate linkage in the PEG protein adduct was studied on samples of modified ribonuclease incubated at 4 and 40°C. The hydrolysis was appreciable at pH 9.5 with a $T_{10\%}$ of 6 d at 4°C and of 1 d at 40°C, but is virtually undetectable at pH 7.4.

Pharmacokinetic Behavior of Polymer-Modified Protein

The effect of enzyme modification by PEG was studied using superoxide dismutase as a model, we are presently studying this enzyme for its antiinflammatory properties (*16*). In Fig. 7, the clearance times of native and of enzyme modified to a different extent is reported. The clearance data are similar to those already found by us for SOD samples derivatized by the cyanuric chloride method (*7*) or when the PEG was bound via active esters after oxidation to the carboxylate of the carbinol function.

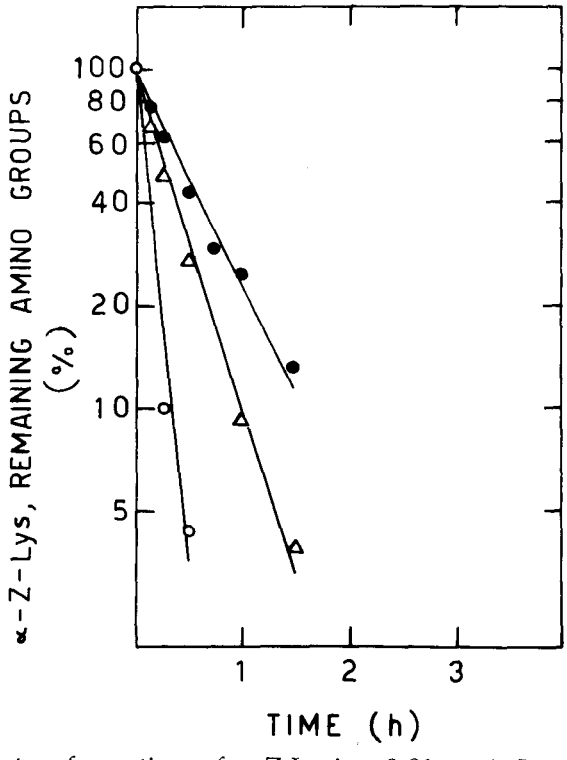

Fig. 3. Rate of reaction of α-Z-Lysine 0.01 mg/mL with PEG-1900-ω-trichlorophenylcarbonate, 60 mg, in phosphate buffer, 0.2*M*, pH 8.3 (•); borate buffer, pH 8.8 (Δ) and pH 9.3 (○).

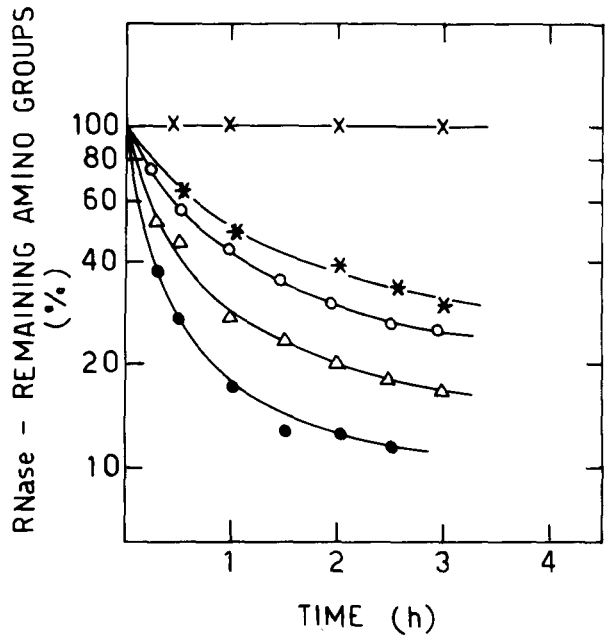

Fig. 4. Rate of modification of ribonuclease-A, 1.4 mg/mL, with PEG-1900-ω-trichlorophenylcarbonate, 28 mg, in phosphate buffer, 0.2*M*, pH 8.3 (○); borate, pH 8.8 (Δ) and pH 9.3 (•), as verified by amino group titration and, at pH 8.8, also by enzymatic activity using cytidine-2′,3′-cyclic diphosphate (x) or ribonucleic acid (*) as substrate.

Recognition of Modified Superoxide Dismutase by Antibodies

By immunodiffusion, the recognition of native enzyme and of samples of the PEG-enzyme with bound 3, 7, 14, and 17 polymer chains was studied. A decrease in the intensity of precipitating bands was found that corresponds to the extent of modification; only in the most extensively modified sample (17 amino groups out of the 20 present in the molecule) did no precipitation occur.

DISCUSSION

Although several methods are thus far available for the modification of proteins by polyethylene glycols, the chemical approach is still valid since previous methods suffer from various disadvantages. These include the toxicity of the chemicals employed, the numerous steps necessary for the activation processes, the low reactivity of intermediates, and finally the easy cleavage of the PEG–protein adduct. The method proposed here, which employs phenylchloroformates as activating reagents, seems to overcome these disadvantaes. The activated PEG is prepared in a single-step procedure, it may be easily titrated and, in spite of its relative stability in neutral aqueous solution, it is reactive enough to modify proteins extensively and in reasonable time periods.

Similar to the results found by Wilchek (*11*) in activated polysaccharidic resins, the PEG *p*-nitrophenylcarbonate is characterized by a higher reaction rate towards protein amino groups than the corresponding 2,4,5-trichlorophenyl derivative. The reagent, however, does not seem convenient because the absorbance of the nitrophenylchromophore hinders the easy and rapid evaluation of the reaction by trinitrophenylsulfonate titration of the unreacted amino groups.

The urethane linkage formed by PEG derivatization appeared relatively stable at the physiological pH of body fluid. Such protein derivatives may therefore be applicable to in vivo studies. The in vitro studies were thus verified by in vivo pharmacokinetics experiments using superoxide dismutase derivatives for which linear kinetics of clearance were obtained.

Because of its interest as an antiinflammatory drug, superoxide dismutase has been one of the model proteins employed to verify the modification procedure. Protein samples containing varying levels of PEG were obtained by stopping the reaction at various intervals of time with an excess of amine. This procedure appeared more reliable than that previously adapted by other authors and also by us (*1*, *7*), which was based on the use of different ratios of reagent and protein at a fixed reaction time. The amount of bound polymer was found critical for both the

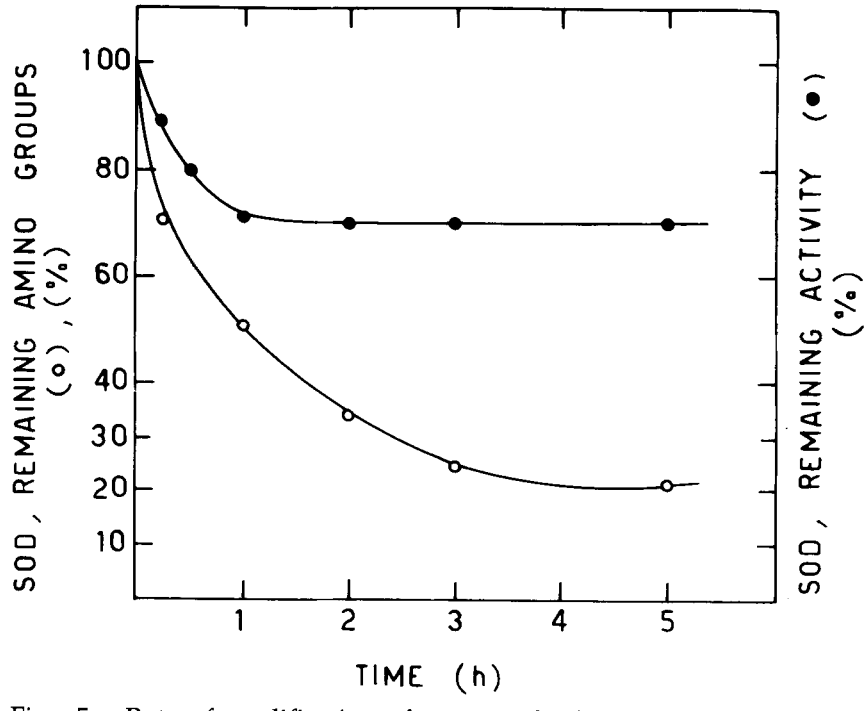

Fig. 5. Rate of modification of superoxide dismutase, 4.2 mg/mL, with PEG-1900-ω-trichlorophenylcarbonate, 40 mg in borate buffer, 0.2*M*, pH 8.8, as verified by amino group titration (○), and enzyme activity (•).

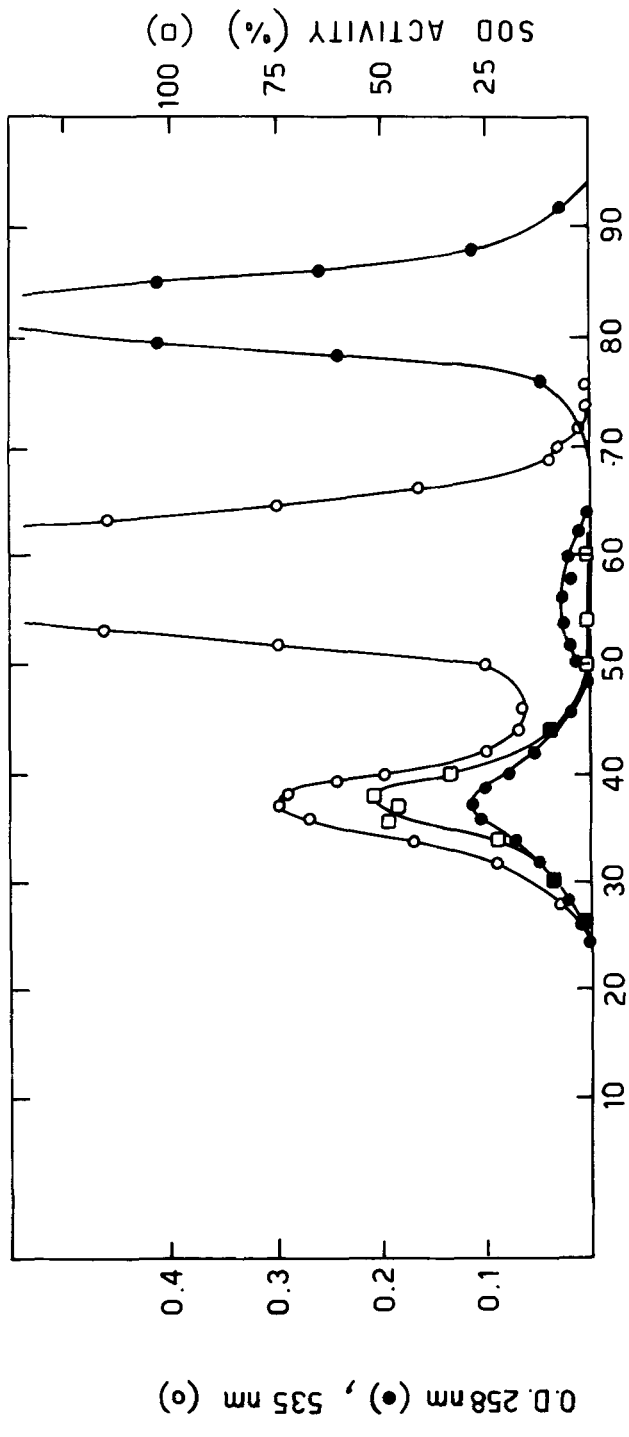

Fig. 6. Gel filtration of a superoxide dismutase sample with 14 amino groups modified with PEG-1900-ω-trichlorophenylcarbonate. The reaction was stopped by addition of excess Gly–Gly. The column used was BioGel-a 0.5 m, 1 × 30 cm. Superoxide dismutase activity (□), OD 258 nm (•), PEG titration with the method of Sims and Shape (○).

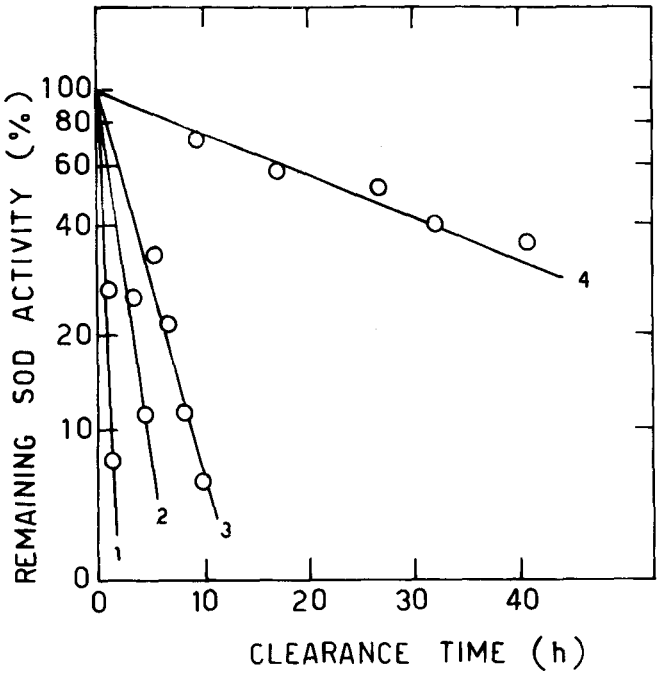

Fig. 7. Plasma clearance of superoxide dismutase activity following intravenous injection of native and PEG-modified superoxide dismutase species into rats. Native SOD (1) and SOD modified with PEG-5000 at three (3), and 17 amino groups (4) and with PEG-1900 at three amino groups (2).

time of clearance in rats and the recognition of the protein by antibodies; however, whereas low levels of modification resulted in large differences of clearance time, the suppression of antibody recognition required a more extensive modification of the available amino groups in the protein molecule.

The modification of superoxide dismutase is accompanied by a limited loss of enzymatic activity, lower than that already observed when cyanuric chloride was used as a coupling agent (*15*). This phenomenon was also observed with ribonuclease, where modification with cyanuric chloride-activated PEG is accompanied by rapid loss of activity as assessed by cytidine-2′,3′ cyclic phosphate whereas the modification with PEG activated with phenylchloroformate leaves the enzyme totally active. This is probably related to the reactivity of PEG–chlorotriazine towards other nucleophiles in the protein, such as that demonstrated for—SH groups (*6*).

With ribonuclease we could observe an interesting, although expected, phenomenon: the difference in enzymatic activity when assessed by a low molecular weight substrate, cytidine-2′,3′-cyclic phosphate and a large molecular weight substrate, ribonucleic acid. Although the modification did not effect the activity with the former substrate, large activity loss was found with the latter. This may be interpreted to result from impaired accessibility to the active site by ribonucleic acid when

part of the protein surface is occupied by the linked polymer. A similar phenomenon was also observed by Koide and Kobayashi on modified elastase (*17*) and by us (*18*) with other enzymes, such as lysozyme, which, upon mofidication, loses its lytic activity towards *Micrococcus leisodecticus* cells.

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REFERENCES

1. Abuchowski, A., Van Es., T., Palczuk, N. C., and Davis, F. F. (1977), *J. Biol. Chem.* **252,** 3578.
2. Abuchowski, A., McCoy, J. R., Palczuk, N. C., Van Es, T., and Davis, F. F. (1977), *J. Biol. Chem.* **252,** 3582.
3. Geiger, B., Von Spect, B., and Arnon, R. (1977) *Europ. J. Biochem.* **73,** 141.
4. Wong, K., Cleland, L. G., and Poznansky, M. J. (1980), *Agent Actions* **10,** 231.
5. Tam, S. C., Blumenstein, J., and Wong, K. (1976), *Proc. Natl. Acad. Sci.* **73,** 2128.
6. Boccù, E., Largajolli, R., and Veronese, F. M. (1982), *Z. Naturforsch.* **38C,** 94.
7. Boccù, E., Velo, G. P., and Veronese, F. M. (1982) *Pharmacol. Res. Comm.* **14,** 113.
8. Buckamann, A. F., Morr, M., and Johansson, G. (1982) *Makromol. Chem.* **182,** 1379.
9. Ehrat, M., and Luisi, P. L. (1983) *Biopolymers* **22,** 569.
10. Beauchamp, C. D., Gonias, S. L., Manapace, D. P., and Pizzo, S. V. (1983) *Anal. Biochem.* **131,** 25.
11. Wilchek, M., and Miron, T. (1982) *Biochem. Int.* **4,** 629.
12. McCord, J. M., and Fridovich, I. (1969) *J. Biol.Chem.* **244,** 6049.
13. Sims, G. E. C., and Shape, T. J. (1980) *Anal. Biochem.* **107,** 60.
14. Snyder, S. L., and Sobocinski, P. Z. (1975) *Anal. Biochem.* **64,** 284.
15. Pyatak, P. S., Abuchowski, A., and Davis, F. F. (1980) *Res. Comm. Chem. Path. Pharm.* **29,** 113.
16. Veronese, F. M., Boccù, E., Schiavon, O., Velo, G. P., Conforti, A., Franco, K., and Milanino, R. (1983) *J. Pharm. Pharmacol.* **35,** 757.
17. Koide, A., and Kobayashi, S. (1983) *Biochim. Biophys. Res. Comm.* **111,** 659.
18. Schiavon, O., and Veronese, F. M., personal communication.