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A NEW CLEAVABLE REAGENT FOR CROSS-LINKING AND REVERSIBLE IMMOBILIZATION OF PROTEINS ‡

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SUMMARY. We have prepared a new bifunctional reagent for the cross-linking and reversible immobilization of proteins through their amine groups. This compound, ethylene glycolyl bis(succinimidyl succinate), reacts rapidly with proteins, at pH 7 and at high dilution. The resulting protein cross-links are readily cleaved at pH 8.5 using hydroxylamine for 3-6 hr. at 37°C. Substantial enzymatic activity was observed with lactic dehydrogenase after such reversible cross-linking. Trypsin immobilized on agarose using this reagent retains full specific activity, is stable for weeks in the cold, and may be released with hydroxylamine at 25°C. This compound appears suitable for studies involving proteins with essential disulfide linkages.

Cleavable reagents for the cross-linking of proteins offer great promise as probes of macromolecular conformation (1-5). In addition, they are especially useful in systems where the reversible immobilization of an enzyme is clearly advantageous (6-8). However, cross-linking reagents that are cleavable by reductants such as dithiothreitol (DTT) are unsuitable for proteins containing essential disulfide bridges. For example, trypsin immobilized on agarose through disulfide-containing linkages and released with DTT, exhibits less thermal stability than the native enzyme (6). Similarly, periodate-cleavable reagents may prove incompatible with proteins containing carbohydrate required for structure or function.

We have recently synthesized a novel bifunctional reagent, for use in the cross-linking or reversible immobilization of proteins through their amine groups. This compound, ethylene glycolyl bis(succinimidyl succinate),

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Abbreviations used: DTT=dithiothreitol; EGS-ethylene glycolyl bis(succinimidyl succinate); THF=tetrahydrofuran; DMF=dimethylformamide; DCC=dicyclohexyl carbodiimide; DCU=dicyclohexylurea; NHS=N-hydroxysuccinimide; SDS=sodium dodecyl sulfate; BAEE=N_α-benzoyl arginine ethyl ester; Hb=hemoglobin; HMD=hexamethylenediamine.

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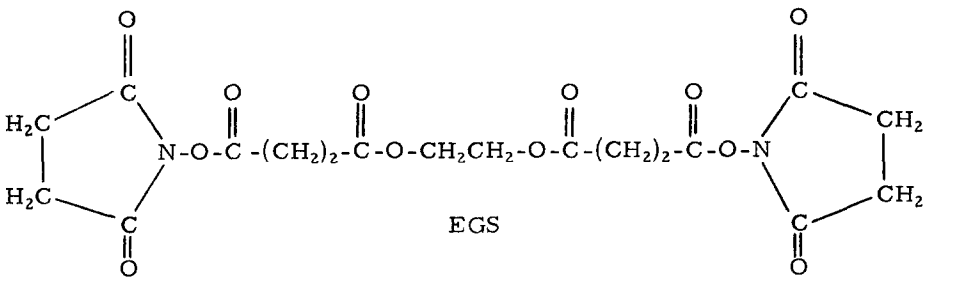


EGS

EGS, may be cleaved under mild conditions with a nucleophile such as hydroxylamine. In this report, we describe the synthesis and properties of EGS, which, we believe, exemplifies a new class of cleavable cross-linking reagents suitable for proteins containing essential disulfide bonds.

MATERIALS AND METHODS

Hemoglobin (twice-crystallized, bovine-type I, lot no. 63C-8000) and lactic dehydrogenase (E. C. 1. 1. 1. 27; rabbit muscle-type II, lot no. 77C-9570) were obtained from Sigma Chemical Company. Trypsin (E. C. 3. 4. 4. 4; twice-crystallized, bovine, lot no. TRL 35D888) was purchased from Worthington Biochemical Corporation. Pharmacia supplied Sepharose ^{R} CL-4B (lot no. 3771). Dimethyl formamide and dioxane were distilled over tosyl chloride and sodium metal, respectively. All other chemicals were of reagent grade or better.

Synthesis of Ethylene glycolyl bis(succinimidyl succinate), EGS. The parent diacid, ethylene glycolyl disuccinate, was prepared from succinic anhydride (2 eq.) and ethylene glycol (1 eq.) in refluxing tetrahydrofuran (THF) overnight with dimethylbenzylamine as catalyst. HCl-acidification of the cooled, water-soluble oil (amine salt) remaining after THF removal (under vacuum), followed by water-washing and recrystallization from ethyl acetate, yielded white crystals with m.p. 73^{0}C. Equivalent weight by titration was 131.5. IR and NMR spectra conformed to the expected structure.

The final product was synthesized by the methods of Anderson et al. (9). To 10.5 g (0.04 mol) of diacid and 10 g (0.08 mol) of N-hydroxysuccinimide dissolved in 300 ml of anhydrous DMF at 0^{0}C were added dropwise, with stirring, 18 g (0.08 mol) of DCC in 25 ml of DMF, and the resulting mixture was stirred overnight. The residue remaining after concentration of the DCU-free filtrate was dissolved in ethyl acetate, re-filtered, and precipitated with n-hexane. After filtering, washing (in ethyl acetate: hexane, 1:1), and drying, the yield of white product was 16.5 g (90% yield) with m.p. 128-133^{0}C. IR and NMR spectra were again consistent with the expected structure.

Hydrolysis of EGS. Aliquots of a solution of EGS in dry DMF were added to buffered solutions (acetate, phosphate, or borate) in the pH range 6.0-9.0. Rates of hydrolysis were monitored either by the increase in A_{260} (viz., NHS release) or by base-titration in the pH-stat. In the spectrophotometric method, corrections were made for the apparent decrease in the extinction coefficient of NHS at 260 nm with decreasing pH in the range examined. These corrections prompted a spectrophotometric determination of the pK_a of N-hydroxysuccinimide (Pierce Chemical Co., lot no. 10197.35). The pK_a measured at 24^{0}C has a value of 5.8 (Figure 1).

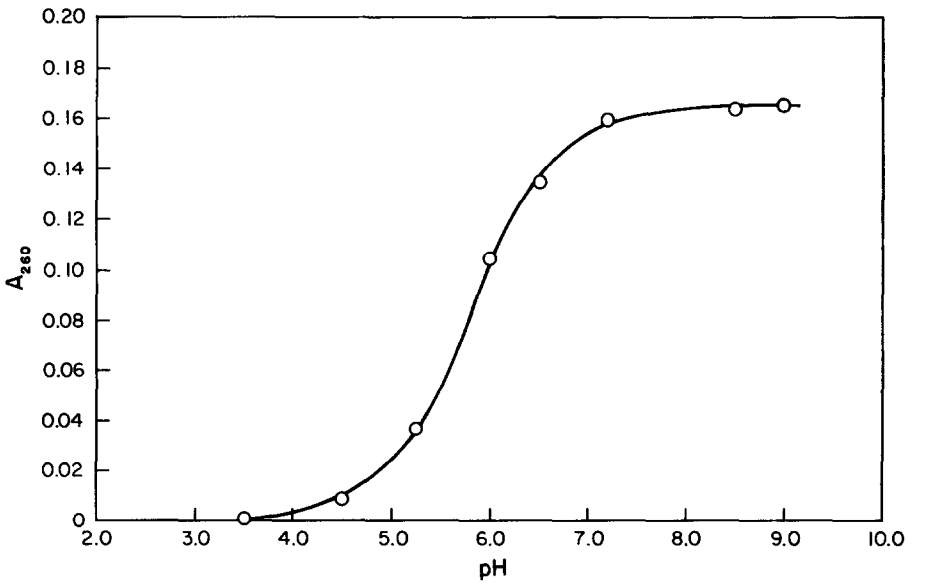


Figure 1. Spectrophotometric titration of N-hydroxysuccinimide. Conditions: [NHS] = 1.82 x 10^{-5}M; temperature = 24°C

Protein Cross-linking. A modification of the method of Lomant and Fairbanks (4) was employed. EGS in dry DMF was diluted with 10 or 100 vol. of 5-10 mM sodium phosphate (pH 7.0) at room temperature. The clear solution was chilled in ice and added to an equal volume of protein solution (0.4-2.0 mg/ml) in the phosphate buffer in the cold (5°C). [Oxamate (4 x 10^{-4}M) and NADH (4 x 10^{-5}M) were included in samples containing lactic dehydrogenase to block the active site.] At various times, aliquots were quenched with 0.05 M lysine, self-buffered at pH 8.5. Incubation of EGS with lysine, followed by addition of protein, constituted a zero-time control.

Cleavage of Intersubunit Cross-links. To an unquenched portion of the EGS-treated protein solution was added NH_2OH (in phosphate buffer, pH 8.5) to a final concentration of 1 M. The stirred solution was brought quickly to 25 or 37°C, and aliquots withdrawn at 1, 3, and 6 hr. for immediate dialysis against three 21-changes of 5 mM phosphate, pH 7.0.

Assay of Cross-linking and Cleavage. The extent of cross-linking and of cleavage were measured by SDS polyacrylamide gel electrophoresis (10). To aliquots of the quenched or dialyzed reaction mixtures, a buffer concentrate was added to give 1% SDS, 1% DTT, and 10 mM sodium phosphate, pH 7.2. All electrophoresis samples were boiled for 15 min. In experiments with hemoglobin, 13-14 μg of protein were typically applied to gels containing 7.5% acrylamide.

Immobilization and Release of Trypsin. Trypsin was immobilized on hexamethylenediamine-Sepharose CL-4B with EGS according to the procedure of Royer et al. (6), using a 40-fold molar excess of EGS over the concentration of free amines. Release was effected by treatment of the Sepharose-bound enzyme at pH 7.5 and room temperature for 4-5 hr. with 1 M hy-

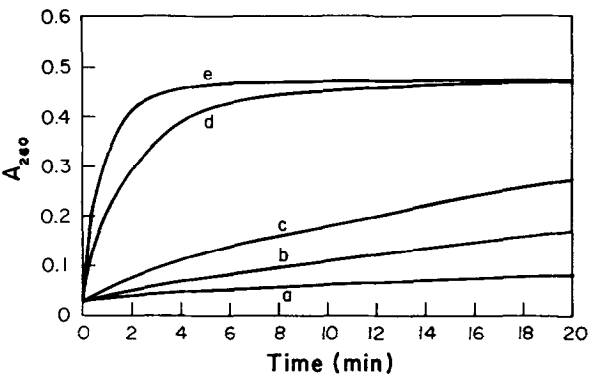


Figure 2. Hydrolysis of Ethylene glycolyl bis(succinimidyl succinate). Conditions: [EGS] = 3 x 10^{-5}M in the following 10 mM buffers: a, acetate, pH 6.0;b, phosphate, pH 7.2; c, phosphate, pH 7.8; d, borate, pH 8.5; e, borate, pH 9.0.

droxylamine in 0.050 M Tris-0.025 M $CaCl_2$, containing 1 mM benzamidine. Native trypsin incubated under identical conditions served as a control. Native and released trypsins were assayed spectrophotometrically according to Schwert and Takenaka (11), using 1 mM BAEE in 0.050 M Tris-0.025 M $CaCl_2$, pH 8.0, 25°C. Bound trypsin was assayed titrimetrically: 50-100 mg damp enzyme conjugate, 20 ml of 1 mM BAEE in 0.5 mM Tris-0.025 M $CaCl_2$, pH 8.0, 25°C.

RESULTS AND DISCUSSION.

A measure of the stability of ethylene glycolyl bis(succinimidyl succinate) at various pH values is provided by the spectrophotometric time courses of hydrolysis shown in Figure 2. These data reflect the release of N-hydroxy-succinimide moieties from the diester. Identical results were obtained titrimetrically, precluding breakdown of the internal alkyl ester linkages. The end points obtained correspond to a 90% purity for this reagent.

As shown in Figure 3a, EGS applied even at high dilution is a very effective cross-linking agent, due to its high reactivity and long solution half-life at pH 7. When hemoglobin at 0.2 mg/ml is incubated with 5 x 10^{-5}M EGS, the major cross-linked product migrates with the molecular weight of a dimer in SDS polyacrylamide gels. The reaction is substantially complete after 20 min. and amounts to modification of about 25% of the subunits present. Higher molecular weight species were frequently observed when the gels were overloaded with protein. In addition, at higher concentrations

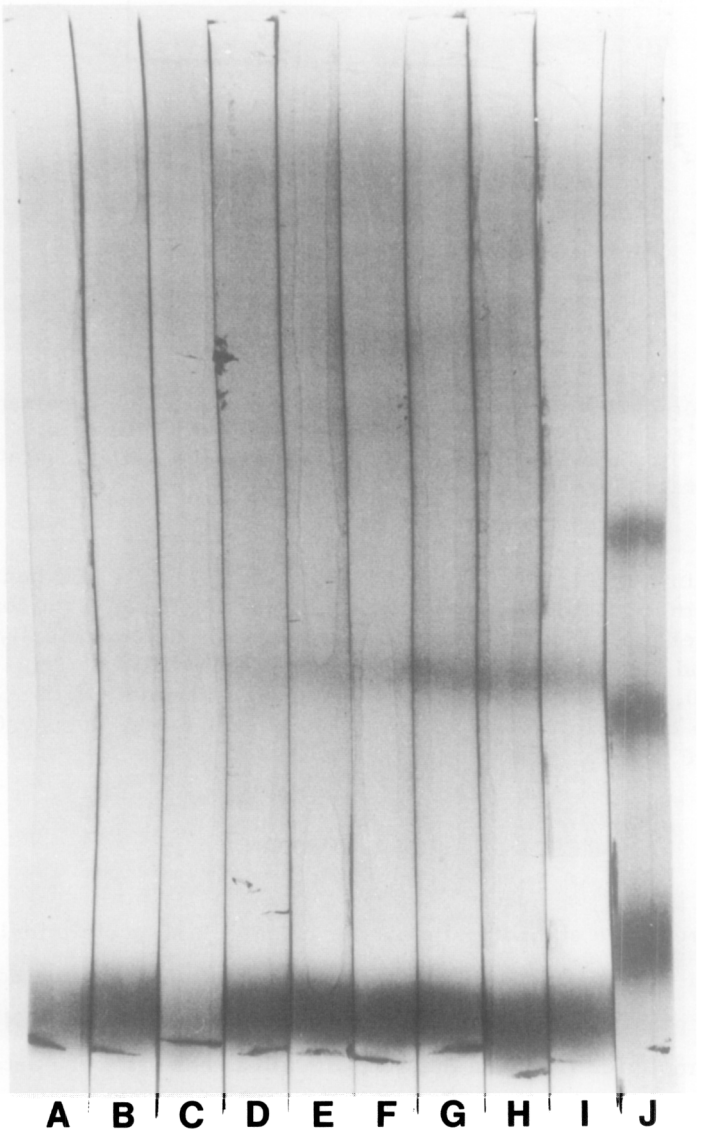


Figure 3a. Cross-linking of Hemoglobin with EGS at 5°C in 5mM phosphate, pH 7.0. A=Hb only; B=0.02 M Lys + 5 x 10^{-4}M EGS, then + 1.0 mg/ml Hb; C,D,E,F,G=0.02 mg/ml Hb + 5 x 10^{-5}M EGS for 5,10,20,30,60 min., followed by quenching with 0.02 M Lys, pH 8.5; H,I = 1.0 mg/ml Hb + 5 x 10^{-4}M EGS for 5 and 10 min., then quenching; J = markers: ribonuclease (13,700), chymotrypsinogen (25,700), and yeast alcohol dehydrogenase (37,000).

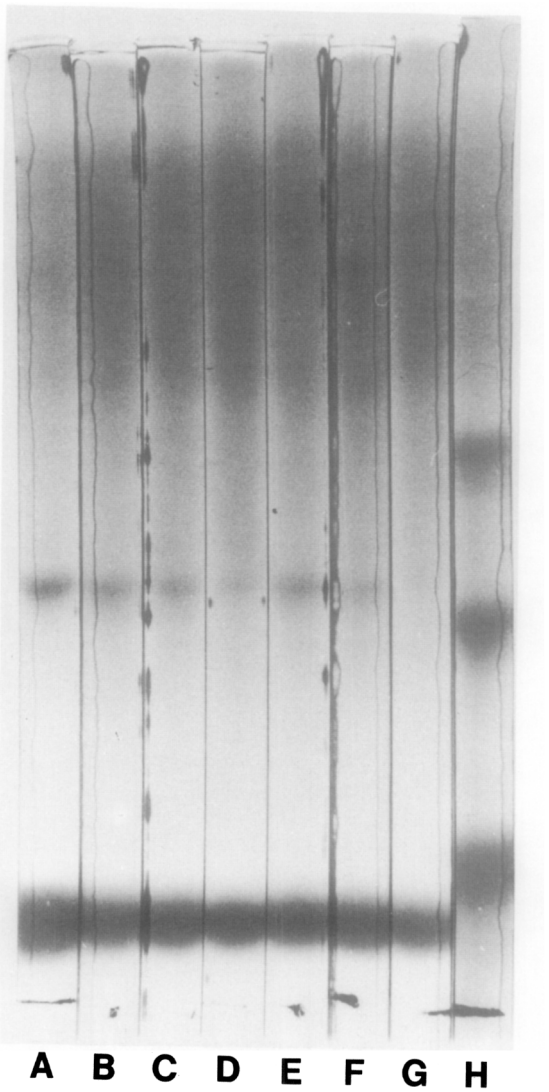


Figure 3b. Cleavage of Hb Intersubunit Cross-linkages with 1 M Hydroxylamine at pH 8.5. A=1.0 mg/ml Hb + 5 x 10^{-4}M EGS for 5 min., followed by quenching with lysine (t_0 for cleavage); B - G = 1.0 mg/ml Hb + 5 x 10^{-4}M EGS for 5 min., followed by incubation with 1 M NH_2OH for 1,3 and 6 hr. at 25°C (B,C,D) or 37°C (E,F,G); H=markers as in Figure 3a.

of reagent, the rate of formation of the dimer species is accelerated. The present results, however, cannot distinguish between inter- and intratetrameric cross-linkages, nor is there apparently any detectable difference between native subunits and monomeric species that are modified, but not cross-linked.

TABLE I. STABILITY OF REVERSIBLY IMMOBILIZED TRYPSIN

Sample	Amine in HMD-Sepharose (μmol/g damp gel)	mg Enzyme immobilized / g damp gel	Specific activity vs. BAEE (units/mg)		
			Initial	After storage*	% of initial
Native enzyme	———	———	38.0	———	———
Immobilized #1	0.416	0.468	37.8	34.8	92
Immobilized #2	0.063	0.131	41.2	39.6	96

* Stored at 5^{0}C in 0.025 M $CaCl_2$, pH 7.0, containing 1 mM benzamidine, for 15 days.

TABLE II. RELEASE OF IMMOBILIZED TRYPSIN WITH HYDROXYLAMINE

Immobilized Preparation no.	Activity vs. BAEE (units/ml)			
	Total Initial	After NH_2OH treatment*		
		Gel-bound	Supernatant	% Released
#1	1.64	0.77	0.35	21
#2	1.67	0.53	0.52	31
Control† (native trypsin)	38.4	____	34.7	95

* Release conditions: 1 M NH_2OH for 5 hr. at pH 7.5, 25°C, in 0.050 M Tris/ 0.025 M $CaCl_2$, containing 1 mM benzamidine.

† Listed as specific activity (units/mg enzyme)

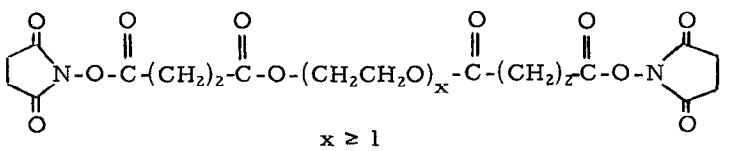
Incubation of the EGS-treated hemoglobin solutions with 1 M hydroxylamine at pH 8.5 results in a gradual loss of material in the dimer region (Figure 3b), reflecting cleavage of both cross-linkages and "end-on" modifications of the protein. After 6 hr., this cleavage appears quantitative at 37°C, and substantially complete even at room temperature. Presumably, stable hydroxamate end-groups are generated on the protein in this process, in addition to carboxylate moieties.

Similar experiments were performed with lactic dehydrogenase, and reversible cross-linking was observed in SDS polyacrylamide gels. Following cleavage, the hydroxylamine-treated enzyme was assayed with NADH (1.5×10^{-4}M) and pyruvate (7.5×10^{-4}M) in 0.033 M potassium phosphate, pH 7.4. Native lactic dehydrogenase incubated with NH_2OH served as a control. The EGS-modified sample (after cleavage) retained 60% of the activity of the native enzyme, as did the control.

Preliminary immobilization experiments were carried out with trypsin on two preparations of hexamethylenediamine-Sepharose CL-4B, as shown in Table I. Very little loss of activity from the gel occurred after extended storage of either preparation at pH 7.0 in the cold, confirming the stability of the glycol ester linkage observed earlier in the hydrolysis experiments on the pure compound. Release of activity from the gel is substantial (Table II), but not complete, after incubation with 1 M hydroxylamine at room temperature (pH 7.5), and is somewhat dependent upon the concen-

tration of amines on the agarose, suggesting multiple linkages to the protein. During cleavage of these alkyl ester linkages, charges generated on the enzyme may result in a shift of the pH optimum, or in the solubility behavior of the enzyme. This may account for the apparent loss of total activity in the samples. Controls indicated no effect of 1 M NH_2OH on the activity of native trypsin. After 6 hours at 25°C, dimeric hemoglobin was still observed on SDS polyacrylamide gels (Figure 3b), reflecting incomplete cleavage. At higher temperatures (e.g., 37°C), significant autolysis prevented evaluation of the extent of release of immobilized trypsin.

In conclusion, the cross-linking properties and cleavability of this new reagent appear promising for work in the soluble state at high dilution. Moreover, EGS appears suitable for reversible immobilization of enzymes, affinity ligands and other biochemicals. In addition, ethylene glycolyl bis(succinimidyl succinate) is the first in a series of similar reagents, having the general structure shown below.



$x \geq 1$

These might be useful for studying structure-function relationships and distances in complex biological structures, such as cell membranes, ribosomes, or nuclei.

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