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New Heterobifunctional Protein Cross-Linking Reagent That Forms an Acid-Labile Link†

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ABSTRACT: A new heterobifunctional protein cross-linking reagent has been synthesized. The reagent is based on 2-methylmaleic anhydride (citraconic anhydride) which forms an acid-labile link upon reaction with amino groups. The second reactive group of the heterobifunctional reagent is a maleimido group. The novel reagent has been used to form a cross-link between two different proteins, a murine monoclonal antibody, J5, and the ribosome-inactivating protein gelonin. Gelonin was first modified by reaction with the anhydride, and the maleimido groups so introduced were allowed to react with antibody that had been modified with 2-iminothiolane to introduce sulfhydryl groups. The conjugate was stable above neutral pH. Incubation of the purified conjugate at mildly acidic pH (pH 4–5) resulted in the release of fully functional native gelonin. The amide bond between the reagent and gelonin was cleaved with about the same sensitivity to acid as that described for 2-methylmaleic anhydride.

Bifunctional cross-linking reagents are extremely useful tools for investigation of the number and arrangement of subunits in complex biological structures (Davies & Stark, 1970; Peters & Richards, 1977; Ji, 1983). The development of heterobifunctional reagents has increased the versatility of protein cross-linking techniques. These reagents are widely used to introduce into proteins various reactive functional groups such as maleimido groups (Yoshitake et al., 1979; Liu et al., 1979), sulfhydryl groups (Traut et al., 1973; Perham & Thomas, 1971), 2-pyridyldithio groups (Carlsson et al., 1978), and azido groups (Ji, 1977). Such materials allow two different proteins to be modified with complementary reactive groups, for example, maleimido groups and sulfhydryl groups, and then subsequently to be cross-linked in a separate reaction that yields only the heterodimer in high yield. Reagents of this type have found widespread use in linking enzymes or toxins to immunoglobulins (Yoshitake et al., 1979; Youle & Neville, 1980; Vitetta et al., 1983) and in the study of biological structures (Rinke et al., 1980; Ji et al., 1980).

Complex structures containing many different polypeptide chains are difficult to characterize when simple bifunctional cross-linking reagents are used since it becomes difficult to identify the components of a cross-linked species on the basis of molecular weight alone. Analysis was made possible by the development of reagents with an easily cleavable bond, and valuable information about the arrangement of proteins in

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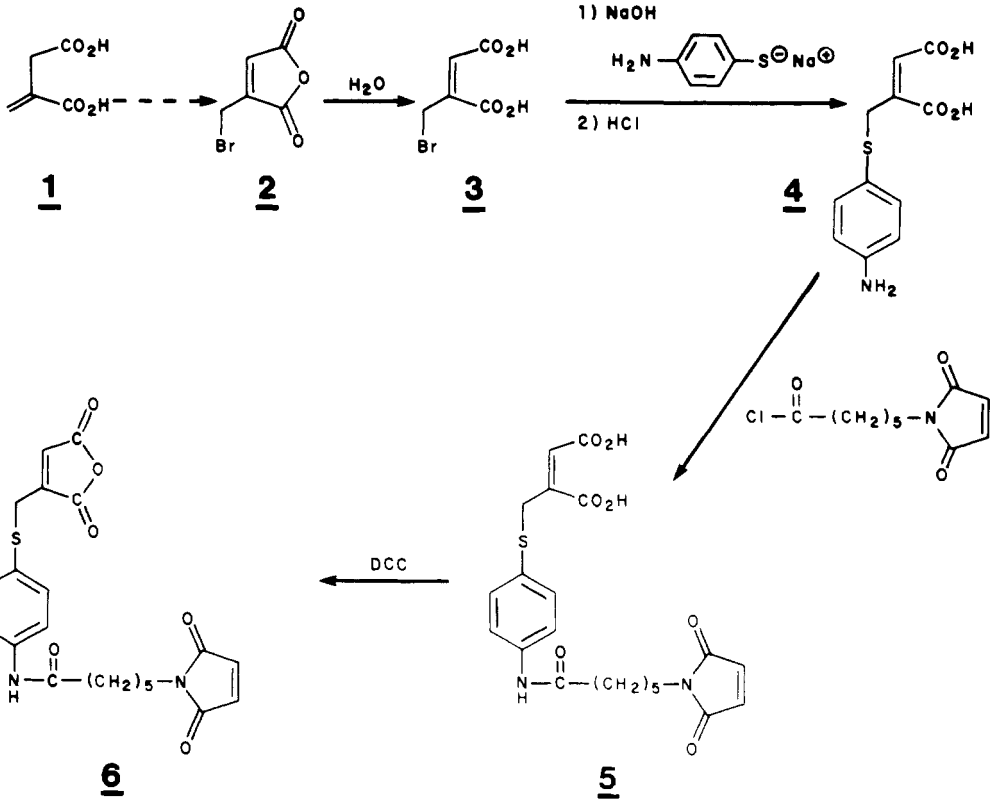
Scheme I: Synthetic Scheme for Heterobifunctional Cross-Linking Reagent 6



ribosomes, membranes, or multienzyme complexes has been obtained, for example, by using cross-linking reagents that contain a disulfide bond (Lambert & Traut, 1981; Wang & Richards, 1974; Lomant & Fairbanks, 1976) or with reagents that contain a *vic*-glycol bond (Lutter et al., 1974; Coggins et al., 1976). Disulfide bonds can be formed between two different proteins in a two-step procedure using the heterobifunctional reagent *N*-succinimidyl 3-(2-pyridyldithio)-propionate (Carlsson et al., 1978). This technique has found widespread use in the many studies on the feasibility of targeting toxins toward specific cells by using specific monoclonal antibodies to cell-surface structures (Vitetta et al., 1983; Edwards, 1983). The efficacy of such conjugates appears to depend on a linkage that can be cleaved (Vitetta et al., 1983; Masuho et al., 1982).

There are drawbacks in the use of these reagents that form cleavable cross-links. Disulfide linkages must necessarily be used under nonreducing conditions in the complete absence of free sulfhydryl groups to prevent disulfide exchange (Lambert et al., 1978). Many enzymes are unstable under such conditions. Periodate oxidation which cleaves *vic*-glycol bonds can also damage proteins, particularly glycoproteins where the carbohydrate residues will be susceptible to oxidation. In all cases, except when a disulfide cross-link utilizes the accessible cysteine residue of one of the proteins (Masuho et al., 1982), the cleavage of the cross-link yields proteins that are still modified at one or more sites.

We have developed a new heterobifunctional reagent that introduces a cross-link between any two proteins and that can be cleaved under mild conditions to yield monomeric proteins one of which is released in native form. The reagent is based on 2-methylmaleic anhydride (citraconic anhydride) which reacts under mild conditions with amino groups of proteins (Dixon & Perham, 1968). The modification is stable above neutral pH but can be easily reversed under mild acidic conditions (pH 4–5) to yield the native protein (Dixon & Perham, 1968; Gibbons & Perham, 1974). We expected that derivatives of the methyl group of 2-methylmaleic anhydride would give compounds that would react with amino groups to yield amides that had similar sensitivity to acidic pH (Kirby & Lancaster, 1970). We have synthesized a maleimido derivative of 2-methylmaleic anhydride and used this novel reagent to cross-link two different proteins. The model cross-linking reaction was between a monoclonal antibody, J5 (Ritz et al., 1980a), and the ribosome-inactivating protein gelonin (Stirpe et al., 1980; Thorpe et al., 1981). Incubation of the conjugate at mildly acidic pH resulted in the release of fully functional native gelonin.

Experimental Procedures

Materials

Seeds from *Gelonium multiflorum* (*Euphorbiaceae*) were obtained from United Chemical and Allied Products, Calcutta-1, India, through the Meer Corp. North Bergen, NJ. Gelonin was purified from the seeds by the method of Stirpe et al. (1980). The murine monoclonal antibody, J5, an IgG_2 that binds to the common acute lymphoblastic leukemia antigen (Ritz et al., 1980a), was purifed from ascites fluid by affinity chromatography on protein A–Sepharose CL-4B as described by Ey et al. (1978). 2-Iminothiolane hydrochloride was from Pierce Chemical Co. L-[U-^{14}C]Cysteine hydrochloride (32.5 mCi/mmol) was purchased from Amersham and diluted to a specific radioactivity of 5 mCi/mmol with nonradiolabeled cysteine hydrochloride before use. A rabbit reticulocyte lysate system for cell-free protein synthesis, which included L-[3,4,5-^{3}H]leucine (specific radioactivity 146.5 Ci/mmol), was obtained from New England Nuclear. Messenger RNA was purchased from Sigma. Betafluor scintillation cocktail was from National Diagnostics (NJ).

Synthesis of New Reagents. The synthetic route for the heterobifunctional cross-linking reagent is shown in Scheme I.

2-[2-(4-Aminophenyl)-2-thiaethyl]maleic Acid (**4**). 2-(Bromomethyl)maleic anhydride (**2**) was prepared according to the method of Laursen et al. (1971) with the modification by Greenlee & Woodward (1980). This material (2 g, 10.6 mmol) was hydrolyzed in water (20 mL) to 2-(bromomethyl)maleic acid (**3**) (1 h, room temperature), and the solution was neutralized with 1 N NaOH (21.2 mL), degassed, and kept under N_2. Meanwhile, 4-aminothiophenol (1.51 g, 12.1 mmol) had been dissolved in degassed 1 N NaOH (11.6 mL) with gentle heating under N_2, and the solution was freed from the insoluble dimer by filtration. The filtrate was diluted with degassed water (12 mL) and added to the above solution of **3**. The final reaction solution was brought to pH 11.0 with 1 N NaOH and stirred under N_2 at room temperature overnight. The solution was then filtered to remove small amounts of bis(4-aminophenyl) disulfide, cooled on ice, and acidified with 1 N HCl, which led to a yellow precipitate. 1-Butanol was added (40 mL), and the mixture was vigorously stirred on ice for 5 min. The solid was then collected by filtration, washed successively with 0.1 N HCl and water, and finally dried in a vacuum desiccator over P_2O_5, to yield **4** in 83% yield (2.23 g, 8.8 mmol). Elemental analysis showed that we had isolated the neutral compound **4**, rather than the hydrochloride salt that one might expect from the above workup procedure: ^{1}H NMR (Me_2SO-d_6)[1] δ 3.56 (s, 2 H), 5.67 (s, 1 H), 6.53 and 7.10 (A′B′, d, J = 8 Hz, 4 H), and 8.30 (br s, 2 H).

Maleimido Diacid **5**. 6-Maleimidocaproic acid was prepared according to the method of Keller & Rudinger (1974) and transformed to its acid chloride with thionyl chloride. 6-Maleimidocaproic acid (1.06 g, 5.0 mmol) was dissolved in dry THF (10 mL) and treated with $SOCl_2$ (0.51 mL, 7.0 mmol), and the solution was refluxed with exclusion of moisture for 1.5 h. The solution was evaporated in vacuo to dryness, and the remaining oil was dissolved in dry THF (5 mL). This solution was added dropwise over 10 min to a solution of **4** (1.16 g, 4.0 mmol) in dry THF (15 mL) and *N*-ethylmorpholine (2.16 mL, 17.0 mmol). After 2 h, the solution was evaporated to dryness under reduced pressure. The remaining oil was taken up into ethyl acetate and the solution washed with cold 0.1 N HCl and then with water. The ethyl acetate solution was dried and the solvent removed by evaporation. The crude product was then purified by flash chromatography on silica gel with $CHCl_3$–MeOH (95:5 v/v) containing 2% acetic acid as eluant. The product was finally passed through a column of Dowex 50 (H^+ form) in MeOH–H_2O (1:2 v/v). Evaporation of the solvent and drying of the oily residue under high vacuum gave a brittle solid foam in 55% yield (976 mg, 2.19 mmol): ^{1}H NMR (Me_2SO-d_6) δ 1.1–1.9 (m, 6 H), 2.30 (t, J = 6 Hz, 2 H), 3.40 (t, J = 6 Hz, 2 H), 3.77 (br s, 2 H), 5.87 (s, 2 H), 6.95 (s, 2 H), 7.26, and 7.56 (A′B′, d, J = 9 Hz, 4 H).

Cross-Linking Reagent **6**. **5** (200 mg, 0.448 mmol) in dry THF (3 mL) was treated at 0 °C with a solution of dicyclohexylcarbodiimide (110 mg, 0.54 mmol) in dry THF (2 mL). The reaction mixture was stirred at room temperature for 45 min and then freed from the precipitated dicyclohexylurea by filtration. The filtrate was concentrated in vacuo to dryness, and the resulting oil was dissolved in dry dioxane (2 mL) and filtered again. The dioxane was finally removed by lyophilization, leaving a brownish solid in quantitative yield: ^{1}H NMR (Me_2SO-d_6) δ 1.1–1.9 (m, 6 H), 2.30 (t, J = 6 Hz, 2 H), 3.15–3.55 (m, 4 H), 6.97 (s, 2 H), 7.46 and 7.68 (A′B′, d, J = 9 Hz, 4 H), and 7.76 (t, J = 1.5 Hz, 1 H).

Methods

The radioactivity of aqueous samples dried onto Whatman glass fiber disks (GFC, 2.4 cm), or precipitated proteins collected onto glass fiber disks, was measured by scintillation counting with Betafluor scintillation cocktail using a Packard Tri-Carb Model 4530 scintillation counter. The efficiency of counting for ^{14}C and ^{3}H was about 90% and 25%, respectively.

Modification of Gelonin with **6** *and* [^{14}*C*]*Cysteine* (*Scheme II*). Samples of gelonin (1.47 mg/mL) in 100 mM sodium phosphate buffer, pH 7.2, were treated at 20 °C for 30 min with varying amounts of **6** in Me_2SO (10–50 μL added/mL of aqueous solution). Each sample was then applied to a column of Sephadex G-25 (superfine) at 4 °C equilibrated with 100 mM sodium phosphate buffer, pH 7.0, containing EDTA (1 mM). The fractions containing the modified protein (**7**) were incubated with excess [^{14}C]cysteine at 4 °C for 30 min and then subjected to a second gel filtration through columns of Sephadex G-25 (superfine) equilibrated in 100 mM sodium borate buffer, pH 8.5. The eluted fractions containing the protein (complex **8**) were analyzed for incorporated radioactivity. In this way, the degree of modification of gelonin with **6** was quantified.

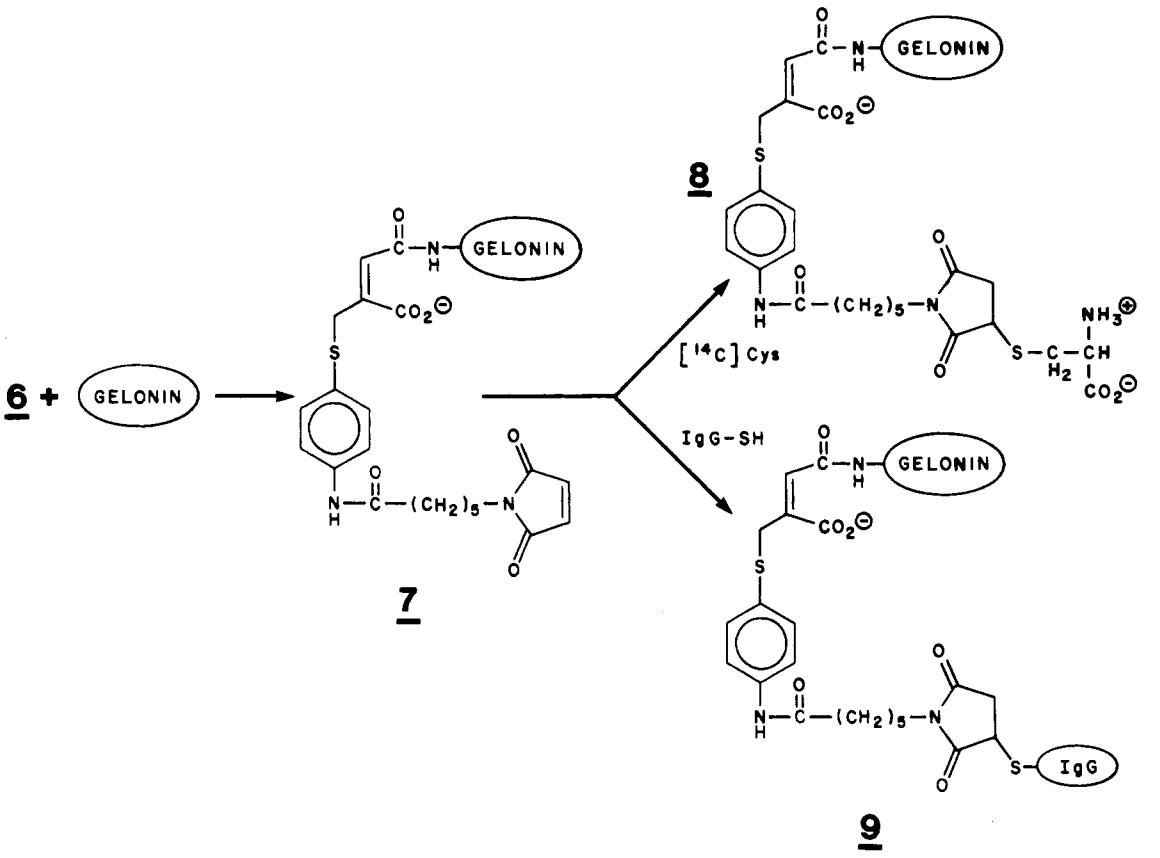
Acid-Catalyzed Release of Radioactivity from Complex **8**. The pH of solutions of modified gelonin **8** in 100 mM sodium borate buffer, pH 8.5, was adjusted to the required value with acetic acid, and the solutions were then diluted with an equal volume of 100 mM citric acid–phosphate buffer (McIlvaine, 1921) at the required pH. In this way, samples having final pH values of 4.0, 5.0, 6.0, and 7.0 were prepared and then incubated at 37 °C. At different times, samples were withdrawn and passed through columns of Sephadex G-25 (superfine) in 100 mM sodium borate buffer, pH 8.5. For each sample, the radioactivity eluting in the protein peak and that released from the protein were quantified.

Modification of Monoclonal Antibody J5 with 2-Iminothiolane. J5 antibody (2 mg/mL) in 60 mM triethanolamine hydrochloride buffer, pH 8.0, containing potassium phosphate (7 mM), NaCl (100 mM), and EDTA (1 mM), was degassed and then treated with 2-iminothiolane (1 mM) for 90 min at 0 °C under nitrogen. Stock solutions of 2-iminothiolane hydrochloride (0.5 M) were prepared as described previously (Lambert et al., 1978). The reaction was terminated by gel filtration at 4 °C through a column of Sephadex G-25 (fine) equilibrated with 5 mM Bistris–acetate buffer, pH 5.8, containing NaCl (50 mM) and EDTA (1 mM). Sulfhydryl groups introduced into the antibody in this way were quantified spectrophotometrically by the method of Ellman (1959).

Conjugation of Modified J5 with **7** *and Purification of Cross-Linked Complex* **9**. Modified J5 (8 mg, 0.05 μmol) in 5 mM Bistris–acetate buffer, pH 5.8 (15 mL), containing NaCl (50 mM) and EDTA (1 mM) was mixed at 0 °C with a 5-fold molar excess of complex **7** (8 mg of gelonin) in 100 mM sodium phosphate buffer, pH 7.0 (11 mL), containing EDTA (1 mM) and then with 0.5 M triethanolamine hydrochloride buffer, pH 8.0 (0.15 mL), to give a final pH of 7.0. The mixture was incubated at 0 °C for 2 h, and then freshly prepared *N*-ethylmaleimide (1 mM) in ethanol (0.26 mL) was added to block any remaining free sulfhydryl groups. After 30 min at 0 °C, the solution was maintained on ice while concentrating to 13 mL by using an immersible ultrafiltration unit (Millipore Corp., CX-10 filter). The mixture was then applied to a column (95 cm × 2.6 cm) of Sephacryl S-300 equilibrated at 4 °C with 5 mM sodium phosphate buffer, pH

[1] Abbreviations: Me_2SO, dimethyl sulfoxide; THF, tetrahydrofuran; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol.

Scheme II: Modification of Gelonin with **6** and Subsequent Reaction with [^{14}C]Cysteine or with Immunoglobulin (IgG) Bearing a Free Sulfhydryl Group[a]



[a] For simplicity, only one of the two possible reaction products of type **7** is shown, and only a single molecule of **6** is shown reacting with gelonin.

7.0, containing NaCl (15 mM) and NaN_3 (0.4 mM). Gel filtration separates the conjugate and native J5 (M_r 160 000) from non-cross-linked gelonin (M_r 30 500; Thorpe et al., 1981) and from some high molecular weight aggregates. The major peak, corresponding to a molecular weight range of 160 000–220 000 and shown by polyacrylamide/sodium dodecyl sulfate gel electrophoresis to contain both native J5 and cross-linked conjugate **9**, was pooled and passed through a column (5 mL bed volume) of carboxymethylcellulose (Whatman CM-52) equilibrated in the same buffer. The column was washed with 1 column volume of buffer and the eluants combined. Under these precise conditions of ionic strength and pH, native J5 was bound by the column while cross-linked complexes of type **9** passed through without retention. Monoclonal antibodies are heterogenous with respect to charge (Cowan et al., 1973): the sample of J5 used in these experiments was, prior to modification, passed through a carboxymethylcellulose column under identical conditions. Only the J5 that bound to the column was used in cross-linking experiments. It was eluted from the column with buffer containing 1.0 M NaCl (yield 66%).

The solution containing purified complex **9**, separated from the non-cross-linked monomeric proteins, was concentrated at 0 °C by using an immersible ultrafiltration membrane (Millipore, CX-30), dialyzed against 10 mM potassium phosphate buffer, pH 7.8, containing triethanolamine (0.5 mM) and NaCl (145 mM), and finally stored at 4 °C after sterile filtration through a Millex-GV filtration membrane (0.22 μm; Millipore).

Protein concentrations were determined by absorption at 280 nm assuming $E^{1\%}_{1cm}$ values of 14.0 and 6.7 for J5 and for gelonin (Stirpe et al., 1980), respectively. Reagent **6** has an absorption maximum at 266 nm and has ϵ_{280nm} of 12 000 M^{-1} cm^{-1} in 10 mM potassium phosphate buffer, pH 7.2, containing NaCl (145 mM); allowance must be made for this fact in calculating an extinction coefficient for **9** ($E^{1\%}_{1cm}$ = 13.5 at 280 nm).

Acid-Catalyzed Release of Gelonin from Complex **9**. Samples of **9** (0.54 mg/mL) were diluted 1:2 (v/v) with 100 mM citric acid–phosphate buffer (McIlvaine, 1921) to give final pH values of 4.0, 5.0, and 6.0. The solutions were incubated at 37 °C and samples withdrawn at different times for analysis of gelonin activity and for analysis by polyacrylamide/sodium dodecyl sulfate gel electrophoresis. Samples for the former were diluted 10-fold into 150 mM Tris-HCl buffer, pH 8.8, containing sodium phosphate (9 mM), NaCl (18 mM), and bovine serum albumin (0.2 mg/mL) and were then stored at 4 °C prior to assay; the final pH was 8.5. Samples (50 μL) for polyacrylamide gel analysis were placed on VSWP-025 dialysis membranes (Millipore) floating on 5 mM triethanolamine hydrochloride buffer, pH 7.8, containing potassium phosphate (5 mM) and NaCl (70 mM), and dialyzed for 2 h at 4 °C after which the pH of the samples was 7.8.

Polyacrylamide Gel Electrophoresis. Cross-linking reactions, conjugate purification, and acid-catalyzed release of monomeric proteins were analyzed by polyacrylamide/sodium dodecyl sulfate gel electrophoresis in gel slabs (145 mm × 90 mm × 0.75 mm) cast with acrylamide gradients (5–10%) prepared according to Laemmli (1970). Samples (25 μL) in buffer, pH 7.8, were prepared for electrophoresis at room temperature by mixing with urea (28 mg) and 10% (w/v)

sodium dodecyl sulfate (3 μL) containing iodoacetamide (100 mg/mL). The urea was necessary to ensure complete denaturation of the proteins in the absence of heating, and iodoacetamide was added to react with any free sulfhydryl groups (Lambert et al., 1978). Proteins were stained with Coomassie Blue R-250 (Lambert et al., 1978), and gels were dried onto dialysis membranes for scanning with a Quick-Scan gel scanner from Helena Laboratories. It was assumed that for a given protein there was a linear relationship between the amount of stain and the amount of protein on the gel (Vogel et al., 1972).

Protein Functional Assays. The antigen-binding activity of the J5 antibody was judged by indirect immunofluorescence on Namalwa cells as described by Ritz et al. (1980b).

The activity of gelonin in the inhibition of protein synthesis was measured in a rabbit reticulocyte lysate system. The assay was based on that described by New England Nuclear (Boston, MA) and by Pelham & Jackson (1976). One-microliter samples of gelonin, or of modified gelonin (**7**–**9**), diluted to 0.024 μg/mL with 10 mM potassium phosphate buffer, pH 7.4, containing NaCl (20 mM) and bovine serum albumin (0.2 mg/mL), were added to the reticulocyte lysate (10 μL) in 0.5-mL Eppendorf tubes at 0 °C. After 5 min, the reactions were started by the addition of 16 μL of a mixture containing salts and buffer cocktail (New England Nuclear), a mixture of 19 amino acids as described by Pelham & Jackson (1976), messenger RNA (5 μg/mL), and [^{3}H]leucine (1 mCi/mL) diluted to a specific radioactivity of 57 mCi/μmol. After the solution was rapidly mixed, the tubes were incubated at 30 °C. Samples (3 μL) were taken at different times, and the incorporation of [^{3}H]leucine into protein was quenched by dilution into distilled water (0.4 mL). Radiolabeled protein was quantified as described by Pelham & Jackson (1976).

Results

The strategy for the synthesis of **6** had to avoid two facile reactions of intermediate **3**: isomerization to the corresponding fumarate analogue (Laursen et al., 1971) and substitution via S_N2' reactions rather than the desired S_N2 processes. The former could be prevented nearly entirely by hydrolyzing anhydride **2** in dilute aqueous solutions and neutralizing diacid **3** immediately after complete hydrolysis. The small amount of (bromomethyl)fumaric acid formed (ca. 5%) could be removed at the stage of compound **4**, where the corresponding fumaric acid derivative was soluble in 1-butanol. The product stemming from an apparent S_N2' substitution accounted for about 50% in the product mixture for **4** when the reaction was performed in an aqueous solution at pH 7.0, but its formation could be completely suppressed by raising the pH of the reaction mixture to 11.0 where all the *p*-aminothiophenol is converted to the thiophenolate anion. The key intermediate **4** was used in a straightforward way to synthesize **6** and related reagents. These syntheses will be described elsewhere (B. S. Kuenzi and W. A. Blättler, unpublished results).

Reagent **6** was used to introduce maleimido groups into the ribosome-inactivating protein, gelonin, as represented in Scheme II. Figure 1 shows the linear relationship between the number of maleimido groups introduced into the protein and the initial concentration of **6**. The proportion of reagent that reacted with the protein under these conditions was about 8%.

Complex **8**, made with [^{14}C]cysteine, was used to measure the hydrolysis of the bond formed between gelonin and **6**. Figure 2 shows the release of radioactivity from gelonin when the modified protein was incubated at 37 °C in buffers of different pH. Release was slow at pH 7, with only 8% loss

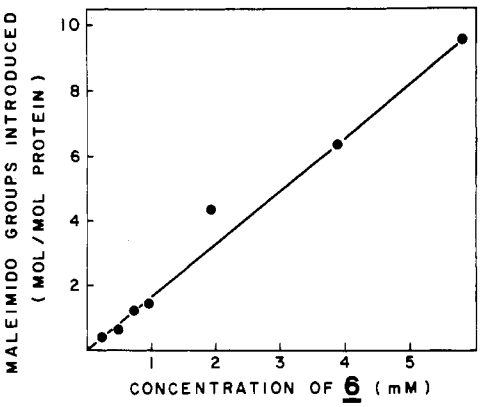


FIGURE 1: Reaction of **6** with gelonin. The reaction was performed as described under Methods. The number of maleimido groups incorporated into the protein at each concentration of **6** was measured by reaction of the product **7** with [^{14}C]cysteine as described under Methods.

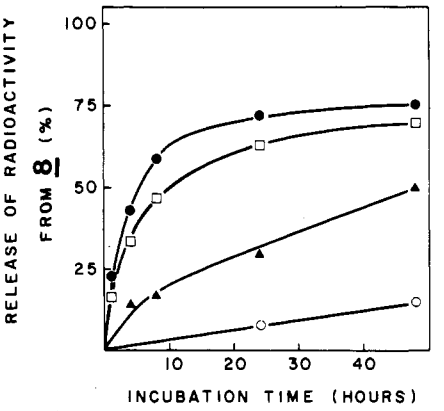


FIGURE 2: Acid-catalyzed release of radioactivity from **8** at 37 °C. Radiolabeled complexes of type **8** (Scheme II) were made by reaction of **7** with [^{14}C]cysteine. Samples of modified protein were incubated in citric acid-phosphate buffers at pH 4 (●), pH 5 (□), pH 6 (▲), and pH 7 (○), and the radioactivity released from the protein was measured as described under Methods.

in 24 h, while at pH 4, 25% of the radioactivity was released in only 1 h. There was no measurable release of ^{14}C from samples of **8** incubated at pH 8.5. The release of reagent from gelonin at pH 4 appeared to reach a maximum of about 80%. Some nonreleasable cysteine can be explained by reaction of the sulfhydryl group of a second cysteine with the double bond of the maleic acid portion of **8** in a Michael-type reaction, to form a succinyl derivative that would not be acid labile (Gibbons & Perham 1970).

The heterobifunctional reagent **6** was then utilized to form a cross-link between two proteins, namely, the monoclonal antibody J5 and gelonin. The antibody was modified with 2-iminothiolane as described under Methods in order to introduce 2.0 mol of sulfhydryl groups/mol of antibody. This derivatized antibody was mixed with a 5-fold molar excess of **7** that had a level of maleimide substitution of 0.7 group per mol of gelonin. The higher level of substitution of J5 increases the yield with respect to the antibody while the low level of substitution of gelonin reduces the amount of high molecular weight aggregates formed in the cross-linking reaction mixture. The final yield of conjugate after gel filtration and carboxymethylcellulose purification was 37% with respect to J5.

Gelonin is a basic protein (Falasca et al., 1982) and normally binds well to carboxymethylcellulose under the conditions used here to purify the conjugate (**9**). However, modification of gelonin with **6** introduces a net charge change of 2– at near neutral pH, and this results in a gelonin-J5 complex

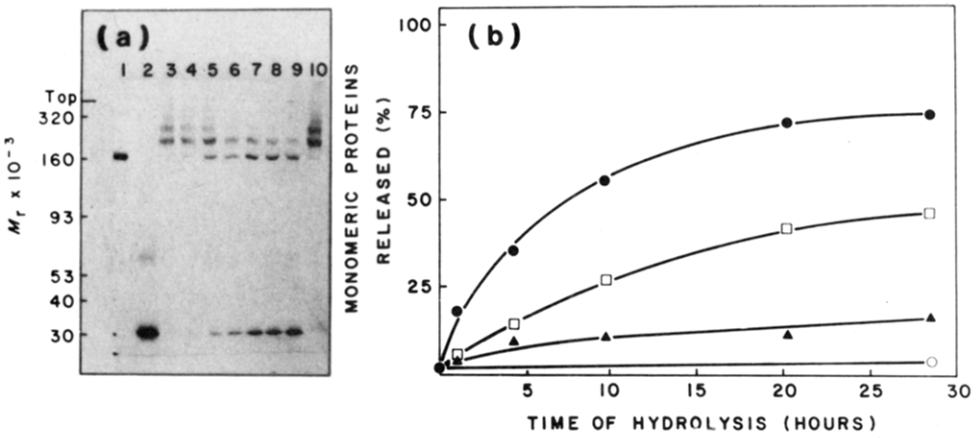


FIGURE 3: Acid-catalyzed release of monomeric proteins from **9**. (a) 5–10% polyacrylamide/sodium dodecyl sulfate gel stained with Coomassie Blue R-250. Lane 1, modified antibody J 5; lane 2, modified gelonin (**7**); lanes 3 and 10, purified J5–gelonin complex **9**; lanes 4–9, complex **9** following incubation at 37 °C in citrate–phosphate buffer at pH 4 for 1, 4, 10, 20, 29, and 125 h, respectively. Molecular weight calibration was based on the mobility of marker proteins of known molecular weight. The relatively weak band, M_r ~60 000, in lane 2 is likely to be a small amount of cross-linked gelonin in the sample of modified gelonin (**7**) used as a marker in this lane. The band was not present in nonmodified samples. (b) Acid-catalyzed release of monomeric proteins from complex **9** estimated by quantitative scanning of polyacrylamide/sodium dodecyl sulfate gels such as that shown in panel a. The ordinate shows the percent of monomeric proteins released (sum of stain associated with the monomeric J5 and gelonin peaks divided by total sum of stain associated with all peaks), and the abscissa shows the time of hydrolysis at 37 °C in citrate–phosphate buffers at pH 4 (●), pH 5 (□), pH 6 (▲), and pH 7.8 (○).

(**9**) that does not bind to carboxymethylcellulose under conditions where *both* unmodified proteins do bind.

Purified complex **9** was analyzed by polyacrylamide/sodium dodecyl sulfate gel electrophoresis, and the results are shown in Figure 3a. The sample in lane 3 shows two major bands with estimated molecular weights of 190 000 and 220 000. A very faint band of estimated M_r 260 000 could also be seen on the gels. The molecular weights of these bands, estimated by their electrophoretic mobility in the gels, were close to values calculated for J5 cross-linked to one, two, and three molecules of gelonin, which are 190 500, 221 000, and 251 500, respectively. Monomeric proteins were virtually undetectable in the sample of complex **9** (lane 3); it was estimated by gel scanning that about 2% of the total staining was associated with non-cross-linked antibody.

Figure 3a also shows the effect of incubating the conjugate in buffer of pH 4 at 37 °C (lanes 4–9). There was progressive loss of the bands corresponding to cross-linked species of type **9** with concomitant appearance of bands that had identical mobility with monomeric J5 (lane 1) and monomeric gelonin (lane 2). The relative amount of protein in the stained bands of polyacrylamide gels such as that shown in Figure 3a can be estimated by gel scanning. Figure 3b shows the results of several such hydrolysis experiments done in buffers of different pH values. Release of monomeric proteins was barely detectable at pH 7.8, while 50% release was achieved after 8 h at pH 4. After prolonged incubation (5 days) at pH 4, 89% of the protein was released as monomeric J5 or gelonin (Figure 3a, lane 9). Comparison of the results shown in Figure 3b with those in Figure 2 shows that the acid-catalyzed hydrolysis of complexes of type **9** was slower than the hydrolysis of samples of **8**, the effect being particularly noticeable at pH 5 and pH 6. The microenvironment of the carboxyl group that catalyzes the hydrolysis of the amide bond (Kirby & Lancaster, 1970) is clearly influenced by the presence of the second protein covalently linked through the maleimido group.

The activity of the J5 component of complex **9** in binding to the common acute lymphoblastic leukemia antigen was compared with that of native J5 as described under Methods. The results showed that the antibody in the complex retained its ability to bind to the antigen. There was no detectable binding to cells that did not possess the antigen.

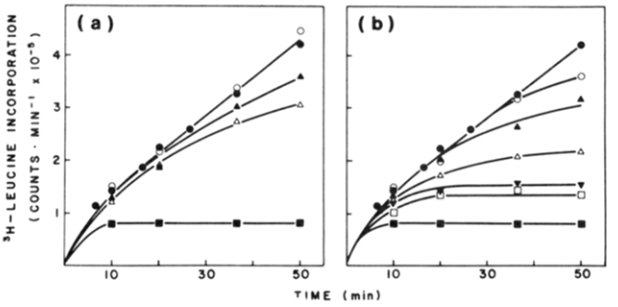


FIGURE 4: Time course of protein synthesis in the presence of complex **9** or in the presence of gelonin. Protein synthesis was measured in a cell-free system from rabbit reticulocytes by the incorporation of [^{3}H]leucine into protein precipitable by trichloroacetic acid as described under Methods. (Panel a) (○) Control assay, total volume 27 μL; (●, ▲, △) assays that contained an amount of **9** equivalent to 0.024, 0.24, and 1.2 ng of gelonin, respectively. (■) Assay that contained 0.024 ng of native gelonin. (Panel b) (●) control assay of volume 27 μL; (■) assay that contained 0.024 ng of native gelonin. The remaining assays contained an amount of **9** equivalent to 0.024 ng of gelonin taken from a sample of **9** incubated in citrate–phosphate buffer, pH 4, after 1 (○), 4 (▲), 10 (△), 21 (▼), and 28 h (□). An assay was also done with native gelonin (0.024 ng) that was incubated at pH 4 for 28 h: the assay was identical with that of native gelonin (■), the difference in values for [^{3}H]leucine incorporation at the same time points being less than the size of the symbols in the figure.

The ability of gelonin to inactivate ribosomes was measured by dilution of samples into an assay of cell-free protein synthesis by using the rabbit reticulocyte lysate system. Figure 4a shows that the ability of gelonin to shut off protein synthesis was drastically reduced in purified complex **9**. While protein synthesis was completely abolished in 7–8 min by 0.024 ng of native gelonin, an amount of **9** corresponding to 1.2 ng of gelonin failed to completely shut off protein synthesis even after 50 min. Other experiments showed that the J5 antibody had no effect on protein synthesis and no effect on the ability of native gelonin to inhibit protein synthesis. Figure 4b shows the inhibition of protein synthesis by samples of **9** corresponding to 0.024 ng of gelonin taken at various times from an incubation of the complex at 37 °C in citrate–phosphate buffer, pH 4. The activity of native gelonin was unaffected by incubation at 37 °C in pH 4 buffer for 28 h. The ability of gelonin to shut off protein synthesis was restored with in-

creasing time of preincubation of **9** at acidic pH, in a manner that paralleled the appearance of monomeric gelonin shown by polyacrylamide/sodium dodecyl sulfate gel electrophoresis (Figure 3). That is, release of gelonin by cleavage of the cross-links to J5 antibody completely restored the toxicity of gelonin.

Discussion

The cross-linking reagent **6** is based on 2-methylmaleic anhydride, a reagent widely used for the reversible modification of amino groups of proteins (Dixon & Perham, 1968; Gibbons & Perham, 1970, 1974). The modification is stable at or above pH 7.5 but is rapidly and completely reversed at mildly acidic pH to restore the native protein (Gibbons & Perham, 1974). The new heterobifunctional reagent reported here reacts readily with proteins to yield derivatives which were stable at pH 8 and which were cleaved under mildly acidic conditions with about the same sensitivity to acid as described for 2-methylmaleic anhydride (Kirby & Lancaster, 1970).

The maleimido group was chosen as a second functional group. This group is widely used in heterobifunctional cross-linking reagents, in part owing to its fast and quantitative reaction with sulfhydryl groups and the stability of the thioether bonds so formed (Yoshitake et al., 1979; Ji, 1983). If the second protein in a two-stage cross-linking procedure contains no cysteine residue accessible to modification, then sulfhydryl groups may be added to the second protein by reagents such as *N*-succinimidyl 3-(2-pyridyldithio)propionate (Carlsson et al., 1978) and 2-iminothiolane (Traut et al., 1973; Lambert et al., 1978), which react with amino groups found on all proteins. The latter reagent was used in the experiments described in this report. The purification of a conjugate formed in high yield between the antibody J5 and gelonin, which could be cleaved under mild acidic conditions, clearly demonstrates the utility of this new cross-linking reagent.

When designing the reagent, we were aware that there can be a side reaction of maleic acid derivatives, where sulfhydryl groups add across the carbon–carbon double bond in a Michael-type reaction. The product of this reaction, and the reaction of the anhydride with an amino group, is a succinic acid derivative that is not susceptible to acid-catalyzed hydrolysis under mild conditions. We anticipated, however, that this side reaction would not be a major problem in forming reversible cross-links between proteins using a reagent of type **6**, since sulfhydryl groups react much more rapidly with maleimides than with maleic acids (Friedman et al., 1949). Gibbons & Perham (1970) reported that some irreversible loss of sulfhydryl groups occurred when aldolase was modified with 2-methylmaleic anhydride, but only when a large excess of reagent was used, sufficient to block greater than 50% of the free amino groups. In the experiment shown in Figure 2, about 20% of the [^{14}C]cysteine introduced into gelonin modified with **6** appeared to be resistant to acid-catalyzed release from the protein. In the cross-linking experiments with J5 and gelonin, the molar concentration of protein-bound sulfhydryl groups was low, about the same as the concentration of maleimido groups of **7** (about 7 μM), and the reaction was done at 0 °C and was terminated by the addition of *N*-ethylmaleimide to block any remaining free sulfhydryl groups. Only 11% of the cross-linked species could not be cleaved to yield monomeric proteins after prolonged incubation at pH 4. Thus, under these reaction conditions, the addition of sulfhydryl groups to the maleic acid portion of the reagent is an unimportant side reaction.

The formation of a conjugate between J5 and gelonin resulted in almost complete loss of the ribosome-inactivating properties of gelonin, despite the fact that the gelonin was modified to give a level of substitution of only 0.7 group per mol so that most of the gelonin in the purified conjugate would have been modified only at a single site. However, on acid-catalyzed hydrolysis of the amide bond formed between **6** and gelonin, native gelonin was released. That this was indeed the case was shown by the complete reappearance of the toxic activity of gelonin, concomitant with the reappearance of monomeric free gelonin.

The new heterobifunctional reagent for protein modification that forms an acid-labile link to one protein should prove to be a useful addition to the armory of cross-linking reagents. Our new reagent can be used in cross-linking studies of biological structures when it is necessary for the analysis that the cross-link be reversible under mild conditions but when the use of reducing agents or periodate oxidation (as required by existing linkers) is undesirable. The reagent can be used to link *any* two species where it is desirable to release under mild conditions one component in its native form. For example, the cleavable cross-link may be between two proteins as described in this paper, between small molecular weight ligands or drugs and proteins, or between polymer supports and proteins or ligands. It is only necessary that one component of the cross-link has an amino group and the second component a sulfhydryl group. The latter can be readily introduced into molecules that contain amino groups by using one of a number of existing bifunctional reagents (Carlsson et al., 1978; Lambert et al., 1978; Ji, 1983).

Last, but not least, this new cross-linking reagent was designed for use in preparing immunotoxin conjugates (Vitetta et al., 1983, Edwards, 1983) such as the conjugate between the J5 antibody and gelonin described in this work. Evidence is accumulating (de Duve, 1983) which suggests that antibodies bound to certain cell-surface antigens enter the cell by the process of receptor-mediated endocytosis. Receptors that are internalized by this mechanism appear to pass through acidified compartments known as endosomes or receptosomes (de Duve, 1983). Thus, it is likely that antibody molecules that enter the cell bound to receptors will be exposed (transiently) to acidic pH. The formation of an acid-labile link between a suitable antibody and a toxin may allow the release of the toxin once inside the cell. We are now investigating the biological properties of conjugates, including that between the J5 antibody and gelonin, in order to establish the utility of the new cross-linking reagent for preparing immunotoxins.

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Registry No. **2**, 26735-82-0; **3**, 32319-83-8; **4**, 94891-01-7; **5**, 94891-02-8; **6**, 94891-03-9; 4-aminothiophenol, 1193-02-8; maleimidocaproyl chloride, 82333-93-5; 2-iminothiolane, 6539-14-6; gelonin, 75037-46-6.

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