

Radioactive Labeling of Protein Carboxyl Groups on Factor VIII: Use of Carbodiimides For Nuclear Medicine

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*Carbodiimides have been used to study macromolecular structure and to produce immunologically active antigens. We have used this method to label a labile coagulation protein, factor VIII, with ^{14}C -glycine-ethyl-ester. No discernible chemical change, loss of biologic function *in vitro*, or alteration of the plasma disappearance of factor VIII resulted. The carbodiimide labeling method has potentially broad application because many biologic molecules contain carboxyl groups that are generally not critical to their chemical or immunologic character. This method can be used to incorporate short-lived positron emitters, such as ^{11}C and ^{13}N , into biologic compounds, or to attach ligands to useful antibodies for subsequent chelation to radioactive metals, such as ^{111}In . Carbodiimides are especially useful for radionuclidic labeling of labile proteins because of the mild conditions, rapid reaction, and firmly attached label.*

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Carbodiimides have been used to produce modifications of carboxyl groups in the study of macromolecular structure (1-3), and to produce precipitating antigen-antibody systems by forming hapten-protein conjugates (4). Heretofore carbodiimides have not been used as a means of introducing exogenous radioactive labels into biologic substances. Since the carbodiimide-labeling method offers the advantages of a mild, simple, and quick reaction, which provides an opportunity for attaching a variety of radionuclides to biologic compounds, it has potentially broad application in nuclear medicine. With this method a covalent bond can be formed between available carboxyl groups and radioactive nucleophiles. By this reaction, we have firmly attached a radioactive tracer to a labile protein, factor VIII (antihemophilic globulin), without measurably changing its biologic activity. In contrast, this protein could not be labeled by any of the standard methods of iodination without complete loss of biologic activity. Recently other methods of labeling have been

devised that either decrease the damage to proteins or increase the variety of radioactive nuclides that may be attached (5,6). These methods are either limited to the use of radioiodine or require a reaction time of several hours.

Radionuclide labeling of factor VIII, employing a water-soluble carbodiimide, and the subsequent chemical and biologic characterization of the labeled protein, provide a suitable model to illustrate this potentially important method.

MATERIALS AND METHODS

The procedure for incorporating a radioactive label into a protein molecule by means of the carbodiimide reaction is illustrated in Fig. 1 and is described as follows. The purity of the protein to

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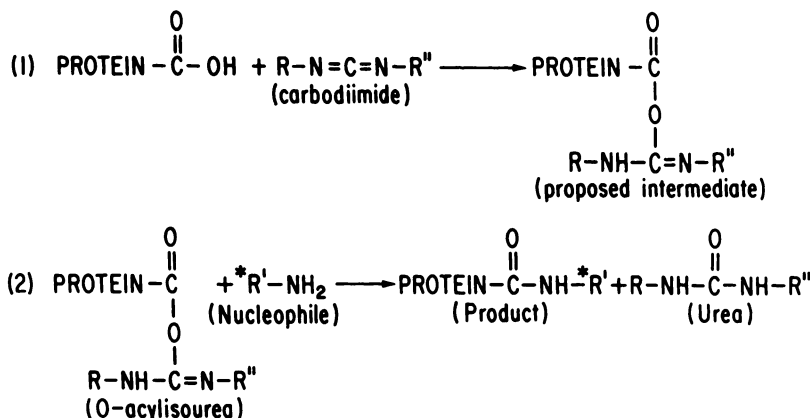


FIG. 1. Carbodiimide reacts with available carboxyl groups of a protein to form an activated intermediate, which then reacts with a radioactive molecule that contains a nucleophilic electron-donating group, i.e., RO^- , RCO_2^- , or RNH_2 . Resulting reaction produces a radioactive label attached to a protein by a covalent bond. (R = ethyl; ${}^*\text{R}'-\text{NH}_2$ = any radioactive amine; R'' = 3-(3-dimethylamino-propyl).)

be labeled, the stability of the label, and the effect of the label on chemical structure and biologic activity of the protein, had to be established prior to its clinical and investigative application. The methods for meeting these requirements were the following.

I. CHARACTERIZATION OF THE PROTEIN TO BE LABELED

A. Chemical Analysis

1. Cellulose Acetate Electrophoresis

Unlabeled factor VIII was purified in our laboratory by the method of Hershgold et al. (7) and was dissolved in saline citrate (0.13 M NaCl, 0.014 M Na citrate, pH 6.5). Electrophoresis was carried out on cellulose acetate strips for 45 min at 250 V in 0.05 M sodium barbital buffer, pH 8.6. Human plasma was diluted 1:3 in saline citrate and processed similarly as a control. The cellulose acetate strips were fixed in 20% sulfosalicylic acid for 2 min, stained for 10 min with Coomassie blue dye* (8), and decolorized in distilled water for 2–3 hr.

2. Gel Electrophoresis

Electrophoresis of unlabeled factor VIII was performed in 0.9% agarose gel plates using 0.05 M sodium barbital buffer, pH 8.6. Twenty-five microliters of protein (0.1 mg/cc) in saline-citrate buffer were applied to the well, and electrophoresis proceeded at 600 V and 14 mA/plate for 2.5 hr. A series of 1-cm strips were then cut in parallel with the origin and these were divided in half longitudinally along the length of the slide. One half of each strip was re-

moved, centrifuged for 5 min at 5 g, and the supernatant assayed for factor VIII activity. The other half of the gel strip was fixed in 20% sulfosalicylic acid for 5 min, stained with Coomassie blue dye for 20 min, and decolorized in distilled water.

The protein stained by Coomassie blue was then compared with the factor VIII assay profile.

3. Gel Column Chromatography

Three milligrams of unlabeled factor VIII were loaded on a 24×2.5 -cm column ($V_0 = 15$ ml, $V_t = 120$ ml) of 2.5% agarose gel and eluted at 20 cc/hr using saline-citrate buffer, pH 6.5. One milliliter fractions were collected and the amount of protein in each tube was measured by optical density at 280 nm. The amount of factor VIII in each fraction was determined by the one-stage factor VIII assay method (7,9).

B. Biologic Function In Vitro

The one stage factor VIII assay (7,9) was done (A) on the original purified protein, (B) on the supernatant from the gel electrophoresis strips, and (C) on each fraction from the gel chromatography column. This assay is essentially a measurement of the degree to which an unknown sample shortens the cephaloplastin-activated clotting time of hemophilic (factor VIII deficient) plasma. The results are expressed as a percent of the activity (clotting-time shortening) in the same volume of normal plasma.

II. LABELING PROCEDURE

A. Labeling

1. Factor VIII, purified as previously dis-

cussed, was added to a test tube in a concentration of 1 mg/ml in 0.03 M NaCl.

2. 0.1 ml of ^{14}C -glycine-ethyl-ester† (27 $\mu\text{Ci}/\text{mg}$), in a concentration of 3.7 mg/ml, was added to each milligram of protein to be labeled. The molar ratio of factor VIII to glycine-ethyl-ester thereby became 1:10⁴.
3. During continuous and vigorous mixing, 0.1 ml of freshly prepared solution of the carbodiimide ECDI-HCl (1-ethyl-3-[3-dimethyl-aminopropyl] carbodiimide HCl)‡, 0.005 M, was then added per mg of protein (1500 ECDI/molecule of factor VIII).
4. The reaction mixture was then incubated at room temperature for 5 min.

B. Purification by Separation

The reaction mixture was purified by passage through a 2.5×24 -cm column ($V_0 = 15$, $V_t = 120$ ml) of 2.5% agarose gel eluted with saline-citrate buffer, pH 6.5, at the rate of 20 ml/hr. One milliliter fractions were collected and their optical density determined at 280 nm. The ^{14}C -glycine-ethyl-ester eluted at the bed volume.

C. Liquid Scintillation Counting

1. A 0.1-ml aliquot from each fraction from the agarose column was solubilized for 5 min at room temperature with 0.5 ml solubilizer.
2. A 10-ml aliquot of scintillation fluid (5 gm PPO and 0.25 gm POPOP in 1 liter of toluene) was added to each sample and radioactivity determined using a liquid-scintillation spectrometer. Quench correction was made after adding a standard source of radioactivity to each sample (10).
3. The distributions of the amounts of radioactivity and protein were plotted and compared.

III. CHARACTERIZATION OF THE LABELED PROTEIN

A. Chemical Analysis

1. *Cellulose Acetate Electrophoresis*
Electrophoresis of labeled factor VIII was performed in paired strips in a manner identical to that previously described in section I-A. After electrophoresis, one strip was stained as described. The other was fixed by drying and the distribution of radioactivity de-

termined using a radiochromatographic strip scanner. The profile of the radioactivity was compared with the profile of the stained protein.

2. *Gel Electrophoresis*

Gel electrophoresis of the labeled factor VIII was identical with that described in section I-A of this paper. Strips from parallel slides were either assayed for their factor VIII activity or counted in a liquid-scintillation spectrometer, as described in section II-C. The movement of the radioactivity and factor VIII activity were compared.

3. *Gel Column Rechromatography of ^{14}C -labeled Factor VIII*

At 1 hr and 24 hr after radioactive labeling, factor VIII was rechromatographed through a 2.5×24 -cm column of 2.5% agarose gel eluted with saline-citrate buffer. One-milliliter fractions were collected, and the optical density at 280 nm and the radioactivity were determined for each fraction.

B. Biologic Analysis

1. *In Vitro*

The one stage factor VIII assay method (7) was used, this being a modification of the activated partial thromboplastin time of Rapaport (9). See section I-B.

2. *In Vivo*

Two milliliters (0.3 μCi) of chromatographically purified, ^{14}C -labeled factor VIII were injected into the ear veins of white rabbits. During the initial 48 hr after injection, a minimum of eight venous blood samples were withdrawn from the other ear into EDTA. Aliquots of 0.2 ml plasma from each sample were mixed with 0.5 ml solubilizer and 10 ml of scintillation fluid, as described in section II-C. The radioactivity was measured using a liquid-scintillation spectrometer.

RESULTS

I. CHARACTERIZATION OF THE PROTEIN TO BE LABELED

A. Chemical Analysis

1. *Cellulose Acetate Electrophoresis*

Factor VIII migrated anodally as a single band in the alpha-3 region (between alpha 2 and beta globulins; reference: human plasma). There was no evidence of any other protein.

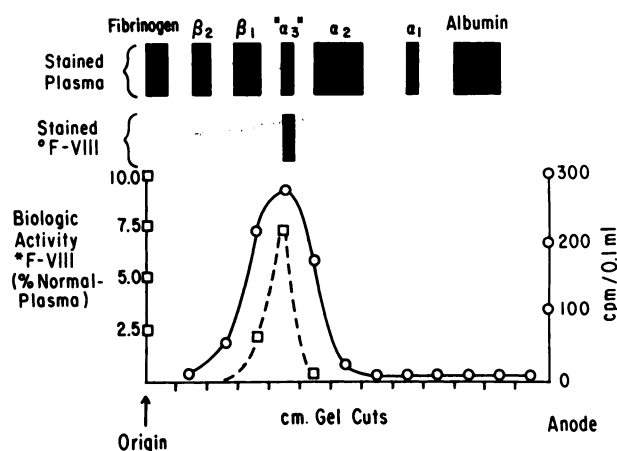


FIG. 2. Agarose-gel electrophoresis of unlabeled (0 F-VIII) and labeled (14 F-VIII) factor VIII. Radioactivity and biologic activity (expressed as percent of normal plasma) were determined on each gel cut. This was compared with stained reference plasma and unlabeled factor VIII run concurrently. Radioactivity, biologic activity, and the alpha-3 protein band (factor VIII) migrate identically.

2. Gel Electrophoresis

Factor VIII migrated as a single band between alpha 2 and beta lipoproteins (reference: human plasma), as detected by staining with Coomassie blue dye and by assay for clotting activity (Fig. 2).

3. Gel Column Chromatography

Gel-filtration chromatography of factor VIII produced a single peak as assessed by optical density (Fig. 3). This peak was within the included volume ($V_0 = 15$ ml, $V_t = 120$, $V_e = 24$ ml). There was no evidence of more than one molecular species by this sensitive method.

B. Biologic Activity In Vitro

One milligram of purified factor VIII had 50 times the biologic activity of 0.1 ml of human plasma and was very close to 100% pure (7). (Reference 7 is a detailed evaluation of the same factor VIII preparation.)

II. LABELING RESULTS

A. Labeling

Ten microcuries of 14 C-glycine-ethyl-ester were added to the reaction mixture per 0.1 mg of factor VIII. Approximately 7% of the added radioactivity was bound to the protein. This represented an average of 6,000 moles of 14 C-glycine-ethyl-ester-HCl (MW 136) per mole of factor VIII (MW 3 million).

B. Purification by Separation

Unreacted 14 C-glycine-ethyl-ester was separated from factor VIII by elution from the preparative column at a volume of 120 ml.

III. CHARACTERIZATION OF THE LABELED PROTEIN

A. Chemical Analysis

1. Cellulose Acetate Electrophoresis

Labeled factor VIII migrated between the alpha 2 and beta lipoproteins exactly as did unlabeled factor VIII (reference: normal human plasma).

2. Gel Electrophoresis

When labeled factor VIII was subjected to gel electrophoresis, the protein (as followed by Coomassie blue), the coagulation activity (as demonstrated by the factor VIII assay), and the radio-

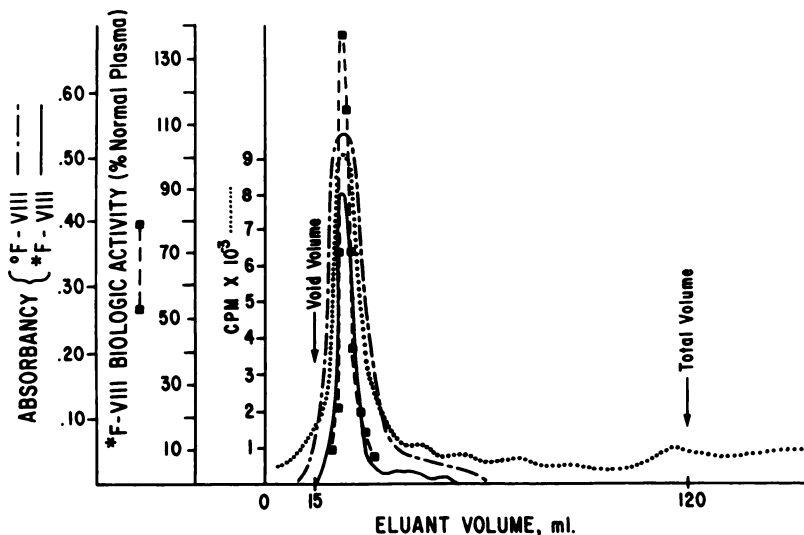


FIG. 3. Rechromatography of labeled (14 F-VIII) and unlabeled (0 F-VIII) factor VIII. (2.5% agarose gel, 2.5×24 -cm column, $V_t = 120$ ml, $V_0 = 15$ ml, V_e [factor VIII] = 24 ml.) No change in the elution pattern before and after labeling. Factor VIII is within the included volume, and no other peak for protein, biologic activity, or radioactivity was detected.

activity migrated together and lay between alpha 2 and the beta lipoproteins as did unlabeled factor VIII (Fig. 2).

3. Gel-Column Chromatography

Labeled and unlabeled factor VIII showed the same elution pattern by 2.5% agarose gel chromatography. There were no other spectrophotometric or radioactive peaks. After 24 hr, repeat chromatography of labeled factor VIII showed the same peak but no free radioactivity (Fig. 3), thus showing the stability of the radioactive label.

B. Biologic Activity

1. In Vitro

The biologic activities of labeled and unlabeled factor VIII were the same.

2. In Vivo

Carbon-14-labeled factor VIII was injected intravenously into 5 rabbits. The half-times for the second phases of the plasma disappearance curves were 10, 10, 15, 16, and 18 hr (Fig. 4). By 10 hr after injection, less than 30% of the injected radioactivity remained in circulation, and by 72 hr the radioactivity level had reached background.

DISCUSSION

The use of carbodiimides as an intermediate activator provides a simple, mild method to label most biologic compounds firmly with either beta or gamma emitters. The reaction involves (A) activation of the carboxyl groups common to almost all biologic compounds by a water-soluble carbodiimide at a neutral pH and room temperature, and (B) the subsequent reaction of the activated carboxyl groups with nucleophiles, such as glycine-ethyl-ester. Reaction is

complete in minutes, making it potentially usable for short-lived isotopes.

Despite the extreme lability of factor VIII, the chemical characteristics of the labeled protein were indistinguishable from those of the unlabeled protein, even though many carbodiimide groups were added to each molecule. Furthermore, the labeling procedure failed to change the biologic activity of the labeled factor VIII—an integral component of the coagulation system—either in vitro or in vivo. The plasma disappearance of the labeled factor VIII in rabbits was similar to reported results in hemophiliacs who were given large amounts of factor VIII and in whom the biologic activity of factor VIII in the plasma was serially assayed (11). There was, therefore, no evidence that the addition of the ^{14}C -glycine-ethyl-ester group on this protein caused any increase in its clearance rate from the plasma.

This mild but effective method of creating a firm bond between a radiolabeled nucleophile and a protein offers multiple possibilities for a broader use of labeled biologic compounds. By means of this chemical activation, a nucleophilic moiety containing either an amine group or a carboxyl group may first be labeled by a relatively harsh or chemically restrictive method and then be firmly attached to the labile protein by use of a mild intermediate carbodiimide activator.

Nucleophilic reagents are electron rich and are attracted to the positive center associated with the carbodiimide. They displace the carbodiimide moiety which is a good leaving group. Common nucleophilic reagents that potentially could be used to effect this displacement are amines, cyanide, alkoxides, and carboxylates. Whether or not these displacements will occur depends on the reactivity of the nucleophilic reagent, which must be capable of reacting more rapidly with the O-acylisourea than does H_2O , which only regenerates the original carboxylic acid group (1). The relative reactivity of each nucleophile will vary, but a quantitative yield is not required since the large differences in molecular size between the labeled protein and the unreacted nucleophile make chemical separation rapid and simple. Harsh oxidizing methods, such as chloramine-T or iodine monochloride (8,9), could therefore be used to iodinate a small moiety having a nucleophilic group in an exposed position, and this gamma-emitting moiety could then be attached to any protein activated by carbodiimide.

With the advent of more sophisticated positron-imaging devices (12), interest in short-lived positron-emitting nuclides is increasing. Carbodiimide intermediates should be helpful in the preparation of ^{11}C and ^{13}N proteins. Carbon-11-labeled carbon

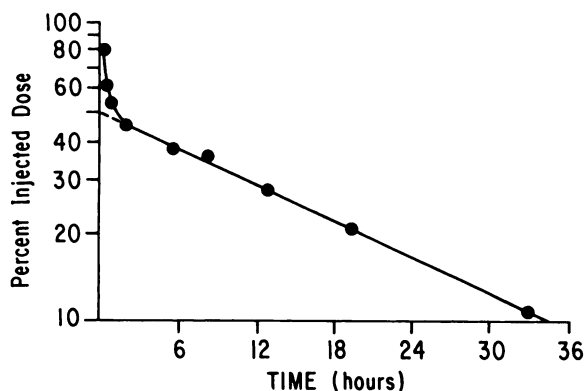


FIG. 4. Example of clearance of radioactivity (labeled factor VIII) from blood of a white rabbit. In this rabbit, biologic half-life of second phase was 15 hr.

dioxide (13), cyanide (14), and glycine (15) have been prepared by direct recoil and/or radiolysis, and the CO₂ can be reduced by lithium aluminum hydride to formaldehyde or methanol (16). Ammonia-N-13 can be prepared indirectly to give yields approaching 500 mCi (17). These radionuclides provide many short-lived nucleophilic groups that could react with carbodiimide-activated proteins.

Although radionuclide-labeled chelates are thermodynamically unstable, they may be very stable kinetically. Indium-111-transferrin provides an excellent example. Since ¹¹¹In has excellent physical properties for clinical use, it may be profitable to attach ¹¹¹In-transferrin by a covalent bond to plasma proteins, such as gamma globulin, using carbodiimide activation. This type of biologic gamma emitter may be useful in developing immunologic tumor-localizing agents.

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FOOTNOTES

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‡ Story Chemical Corp., Muskegon, Mich.

REFERENCES

1. HOARE DG, KOSHLAND DE: A method for the quantitative modification and estimation of carboxylic acid groups in proteins. *J Biol Chem* 242: 2447-2453, 1967
2. RIEHM JP, SCHERAGA HA: Structural studies of ribonuclease XX. Acrylonitrile. A reagent for blocking the amino groups of lysine residues in ribonuclease. *Biochem* 5: 93-99, 1966
3. CARRAWAY KL, KOSHLAND J: Reaction of tyrosine residues in proteins with carbodiimide reagents. *Biochim Biophys Acta* 160: 272-274, 1968
4. GOODFRIEND TL, LEVINE L, FASMAN GD: Antibodies to bradykinin and angiotensin: A use of carbodiimides in immunology. *Science* 144: 1344-1346, 1964
5. BOLTON AE, HUNTER WM: The labelling of proteins to high specific radioactivities by conjugation to a ¹²⁵I-containing acylating agent. Application to the radioimmunoassay. *Biochem J* 133: 529-539, 1973
6. SUNDBERG MW, MEARES CF, GOODWIN DA, et al.: Selective binding of metal ions to macromolecules using bifunctional analogs of EDTA. *J Med Chem* 17: 1304-1307, 1974
7. HERSHGOLD EJ, DAVISON AM, JANZEN ME: Isolation and some chemical properties of human factor VIII. *J Lab Clin Med* 77: 185-205, 1971
8. FAZEKAS DE ST. GROTH S, WEBSTER RC, et al.: Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. *Biochim Biophys Acta* 71: 377-391, 1963
9. RAPAPORT SI, SCHIFFMAN S, PATCH MJ, et al.: A simple specific one-stage assay for plasma thromboplastin antecedent activity. *J Lab Clin Med* 57: 771-780, 1961
10. HENDEE WR: Liquid scintillation counting. In *Radioactive Isotopes in Biological Research*, Hendee WR, ed, New York, Wiley, 1973, pp 199-216
11. POOL JG, ROBINSON J: Observations on plasma banking and transfusion procedures for haemophilic patients using a quantitative assay for antihaemophilic globulin (AHG). *Brit J Haemat* 5: 24-30, 1959
12. PHELPS ME, HOFFMAN EJ, MULLANI NA, et al.: Application of annihilation coincidence detection to transaxial reconstruction tomography. *J Nucl Med* 16: 210-224, 1975
13. WELCH MJ, TER-POGOSSIAN M: Preparation of short-lived radioactive gasses for medical studies. *Radiation Res* 36: 580-587, 1968
14. CHRISTMAN DR, FINN RD, KARLSTROM KI, et al.: Production of carrier-free H¹³Cn for medical use and radiopharmaceutical syntheses. *J Nucl Med* 14: 864-866, 1973
15. FROST D, MICHEL L, SCHWARTZ E: Die radiomarkierung organischer verbindungen durch C¹²(y,n)C¹³-reaktion mittels bremsstrahlung eines 35-MeV-betatron. *Strahlentherapie* 127: 510-515, 1965
16. CHRISTMAN D, CRAWFORD EJ, FRIEDKIN M, et al.: Detection of DNA synthesis in intact organisms with positron-emitting (Methyl-¹³C) thymidine. *Proc Natl Acad Sci* 69: 988-992, 1972
17. VAALBURG W, KAMPHUIS JA, BEERLING-VAN DER MOLEN HD, et al.: An improved method for the cyclotron production of ¹⁵N-labeled ammonia. *Int J Appl Radiat* 26: 316-318, 1975