fluence in a 1:1 mixture of Dulbecco-modified Eagle's and F12 medium supplemented with 5 percent heat-inactivated fetal bovine serum (Reheis Chemical Co.) and 1 percent nonessential amino acids without antibiotics. Cells were rinsed twice in medium without serum, detached in calcium- and magnesium-free phosphate-buffered saline containing 2 m*M* EDTA, and rinsed again in medium without serum before use. The cells were used within ten passages from frozen stocks and were found to be free from mycoplasma contamination [T. R. Chen, *Exp. Cell Res.* **104**, 255 (1977)].
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21. We thank N. Wilson and S. Custead for their assistance. This work was supported by National Cancer Institute grants RO1-CA-28867 (G.L.N.) and RO1-AM-26482 (N.D.F.).
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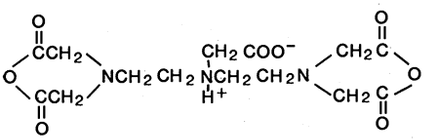
Radioactive Labeling of Antibody: A Simple and Efficient Method

Abstract. *A simple and efficient method of covalently coupling the strong chelator diethylenetriaminepentaacetic acid to proteins was developed for radiolabeling immunoglobulin G antibodies. After being coupled and labeled with indium-111, a monoclonal antibody to carcinoembryonic antigen retained its ability to bind to its antigen in vitro and in vivo. In nude mice with a human colorectal xenograft, 41 percent of the injected radioactivity became localized in each gram of xenograft at 24 hours compared with 9 percent for control antibody and 19 percent for radioiodinated antibody to carcinoembryonic antigen.*

The detection of tumor tissue by radioimmunological methods is receiving considerable attention as a result of the development of hybridoma technology. One assessment is that the use of radiolabeled monoclonal antibody will obviate many of the problems that occur when affinity purified antibody is used for this purpose. A difficulty that will not be alleviated in this way is that of the radiolabel; most investigators continue to use ^{131}I as the externally detectable tracer despite its poor imaging characteristics, somewhat involved labeling procedures, and high degree of instability on antibody in vivo (*1*). Alternative labeling methods have been investigated in which strong chelating groups are covalently attached to proteins so that they may be radiolabeled with metallic radionuclides, often resulting in high stability in vivo. Using a method developed by Krejcarek and Tucker (*2*), Khaw *et al.* (*3*) coupled diethylenetriaminepentaacetic acid (DTPA) to immunoglobulin G (IgG) fragments active against myosin and investigated the localization of the ^{111}In-labeled protein in canine myocardial infarcts. Recently, Scheinberg *et al.* (*4*) used both this method and that of Yeh *et al.* (*5*) to prepare ^{111}In-labeled monoclonal antibody specific for erythroleukemic cells in mice. Although these and other methods provide coupled proteins, they are invariably characterized by complicated syntheses and, most important, by low coupling efficiencies. As a consequence of coupling efficiencies of less than 1 percent, lengthy purification procedures are required to remove hydrolytic products.

We have developed a simple and efficient method of covalently coupling DTPA to proteins by using the bicyclic anhydride of DTPA. The anhydride is prepared by a one-step synthesis and, when protected from moisture, is stable for many months at room temperature. Characterization by infrared spectroscopy, nuclear magnetic resonance (*6*), and mass spectroscopy confirmed the structure of the anhydride to be:



Coupling to antibodies is accomplished as described previously for albumin and fibrinogen (*6*, *7*). A solution of the antibody (typically 20 μl containing 0.3 mg of protein) buffered at *p*H 7.0 with 0.05*M* bicarbonate is added to the solid anhydride (typically 0.7 to 0.9 μg) and the solution is agitated for 1 minute. Coupling efficiency, defined as the percentage of anhydride molecules that covalently attach to the protein, is 70 ± 5 percent when reacted at anhydride-to-protein molar ratios of 1:1 and, under these conditions, the IgG antibodies possess an average of 0.7 DTPA groups per molecule. The efficiency of coupling decreases at *p*H values above or below neutrality, with increasing anhydride-to-

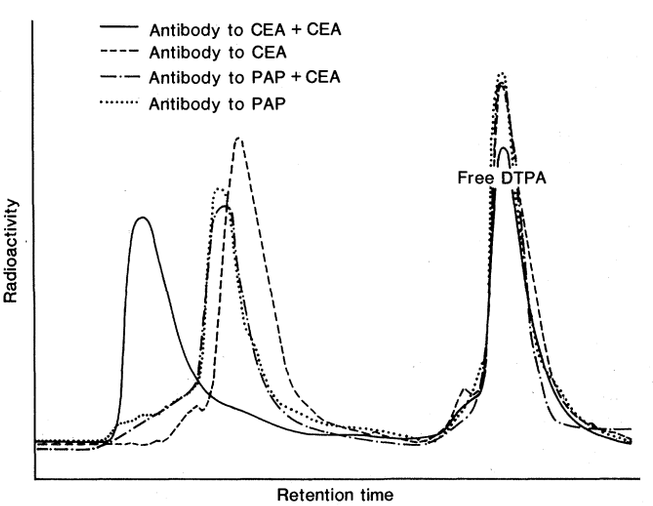


Fig. 1. Radioactivity traces obtained by HPLC analysis of ^{111}In-labeled antibodies to CEA and PAP samples both with and without CEA. Samples with the antibody to CEA consisted of two identical solutions containing 22 μg of labeled antibody (specific activity, 1.2 μCi/μg) in 80 μl of 2 percent bovine serum albumin, 0.05*M* Hepes buffer, and 0.9 percent NaCl. To one sample, 30 μl of a solution of CEA (1 μg/μl) was added and the samples were analyzed. The samples with antibody to PAP were prepared in the same way, except that only 13 μg of labeled antibody was present (specific activity, 3.0 μCi/μg). All four samples were unpurified from free DTPA so that the ^{111}In-labeled free DTPA peak could serve as a marker. Since the molecular weights of CEA and IgG are roughly equal, a large shift to higher molecular weight and shorter retention times is expected in the case where binding with the antigen occurs. This shift to shorter retention time is apparent only in the radioactivity traces for the sample containing antibody to CEA. An identical shift was observed in the corresponding ultraviolet traces.

Table 1. Distributions of monoclonal antibodies to CEA labeled with ^{111}In and ^{125}I and control antibody in nude mice with subrenal implanted tumors known to excrete CEA. The results, obtained 24 hours after injection of the antibodies, are expressed as percentages of the injected dose per gram (wet weight) of tissue normalized to a 25-g animal (mean ± standard deviation).

Tissue	^{111}In		^{125}I	
	Antibody to CEA (N = 6)	Antibody to PAP (N = 6)	Antibody to CEA (N = 5)	Antibody to PAP (N = 5)
Brain	0.25 ± 0.06	0.26 ± 0.14	0.44 ± 0.11	0.25 ± 0.18
Heart	2.09 ± 0.50	2.42 ± 0.79	2.59 ± 0.23	2.33 ± 0.64
Lung	3.84 ± 1.25	4.15 ± 1.92	4.08 ± 0.77	2.79 ± 1.15
Liver	5.52 ± 1.77	6.65 ± 2.51	2.77 ± 0.55	3.61 ± 0.96
Spleen	3.94 ± 0.85	10.9 ± 4.1	2.72 ± 0.43	5.75 ± 2.21
Right kidney	9.25 ± 2.85	7.61 ± 2.49	2.95 ± 0.50	2.55 ± 0.91
Left kidney	7.91 ± 1.51	7.34 ± 2.29	2.91 ± 0.40	2.51 ± 0.88
Tumor	37.9 ± 11.8	9.47 ± 4.81	19.4 ± 6.2	3.98 ± 2.11
Muscle	0.59 ± 0.15	0.53 ± 0.24	0.68 ± 0.01	0.45 ± 0.18
Blood	7.30 ± 0.92	7.19 ± 4.17	11.0 ± 0.7	7.08 ± 4.98

protein molar ratios and with decreasing protein concentration. These observations have applied to each of the polyclonal and monoclonal antibodies investigated. The coupled antibody may be purified from free DTPA by a single passage through a Sephadex G50 gel filtration column. After the addition of ^{111}In in 0.5M acetate buffer, pH 6, to the coupled protein solution, protein labeling occurs quantitatively under normal circumstances. One hour is required for the preparation of coupled, purified, and radiolabeled antibodies.

Control studies, in which deliberate hydrolysis of the anhydride before addition of the protein solution was the only change in procedure, show that no detectable radioactivity became bound to the protein. Such antibodies were examined by high-performance liquid chromatography (HPLC) (*8*). The ultraviolet traces obtained for antibody samples coupled at 1:1 molar ratios show no shift to higher molecular weight with respect to the hydrolyzed control samples, indicating the absence of detectable protein dimer and polymer formation under those conditions.

The ability of antibody coupled with DTPA to bind to its antigen was investigated both in vitro and in vivo with the use of a monoclonal antibody to carcinoembryonic antigen (CEA) and, as a control, a monoclonal antibody to prostatic acid phosphatase (PAP) (*9*). By affinity chromatography an average of 92 ± 2 percent (± standard deviation, N = 5) of the labeled antibody to CEA was retained; for the control antibody this value was 6 ± 3 percent (N = 5). When coupled at a 20:1 anhydride-to-protein molar ratio, an average of 79 ± 7 percent (N = 4) of the labeled antibody was retained. This reduction in retention over antibody coupled at a 1:1 molar ratio presumably reflects the effect on antigen binding of placing an average of five DTPA groups on the molecule.

Antibody integrity was also evaluated by HPLC analysis of the labeled proteins with and without the addition of CEA. As shown in Fig. 1, the retention time of the control antibody to PAP detected by radioactivity was unchanged by the addition of the antigen, whereas a shift toward shorter retention time (that is, higher molecular weight) was apparent in the case of the antibody to CEA and was due to the binding of the antibody with its antigen.

Animal studies with labeled antibody were performed in male BALB/C nude mice with human colorectal cancer xenografts either in the subrenal capsule or in the ear (*10*). The tumor was originally derived from the HT-29 cell line (*11*) and expresses CEA in the nude mouse (*10*). Animals were used in this study 15 to 18 days after implantation when the subrenal tumors weighed about 10 mg and the intrapinnal tumors weighed about 170 mg. Each animal received (by tail vein injection) 0.1 to 0.2 ml of a solution containing, per milliliter, 100 μg of ^{111}In-labeled antibody to CEA or to PAP (specific activity, 0.1 to 3.0 μCi/μg) in 2 percent bovine serum albumin, isotonic saline, and 0.05M Hepes buffer. The animals were killed 24 hours after the injection.

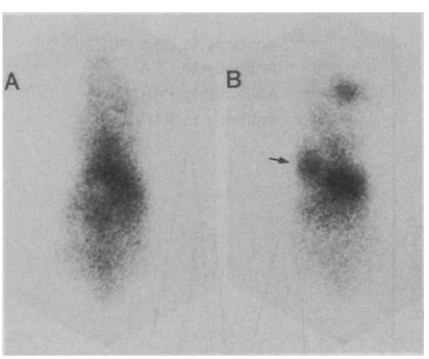


Fig. 2. Gamma camera images of the distribution of ^{111}In-labeled monoclonal antibodies to (A) PAP and (B) CEA in two athymic nude mice, both containing a CEA-expressing tumor in the right ear. The anesthetized animals are shown lying headup on their stomachs; both were imaged to the same number of total counts (200,000) by means of a pinhole collimator. One day before imaging, each animal received an injection (in the tail vein) of 20 μg of antibody (specific activity, 0.6 to 2.0 μCi/μg) in 0.2 ml of 2 percent bovine serum albumin, 0.05M Hepes buffer, and 0.9 percent NaCl. The arrow points to an additional tumor in the left shoulder of the animal that received the labeled antibody to CEA.

As shown in Table 1, the accumulation of ^{111}In-labeled antibody to CEA in the subrenal tumor was four times that of the control antibody. When the results of a duplicate experiment were included, the accumulation in the tumor was 40.5 ± 11.4 percent per gram (± standard deviation, N = 9) for the labeled antibody to CEA and 8.3 ± 4.0 percent per gram (N = 10) for the control antibody to PAP. In each case, the accumulation of the two antibodies in the tumor was significantly different ($P < .001$).

For comparison, we performed a similar study using the same animal model with subrenal tumor implants and antibodies labeled with ^{125}I (*12*). The accumulation of ^{125}I in the tumor was again significantly greater when the label was attached to the monoclonal antibody to CEA rather than to the control antibody ($P < .001$) (Table 1). Furthermore, the ratios of the radioactivity from the antibody to CEA to that from the antibody to PAP were approximately the same for ^{111}In and ^{125}I (4.0 and 4.9, respectively). The increased accumulation of ^{111}In compared with ^{125}I in liver, spleen, and kidneys has been observed by us previously with labeled fibrinogen (*13*) and may be due to differences in the catabolism of the labels in these organs and in the tumor.

Animals with intrapinnal tumors also showed increased radioactivity at 72 hours if they had received the ^{111}In-labeled antibody to CEA (20.6 percent per gram, range 12.9 to 27.3 percent per gram, N = 4) rather than the control antibody (2.7 percent per gram, range 1.7 to 3.4 percent per gram, N = 3).

Images obtained at 24 hours in two of the animals with intrapinnal tumors are presented in Fig. 2. The images show the increased uptake of radioactivity in the ear of the animal that received ^{111}In-labeled antibody to CEA compared with the animal that received labeled antibody to PAP. The tumor could not be visualized in the animals that received ^{67}Ga-labeled citrate as a further control. The

animal in Fig. 2 that received the labeled antibody to CEA had an additional tumor growing subcutaneously below the left shoulder (arrow). This tumor, the result of the accidental deposition of tumor cells from the trocar at the insertion site, is apparent despite the high background activity in liver.

Our results demonstrate that IgG antibodies may be coupled with DTPA simply by reaction with the cyclic anhydride in aqueous solution and that the labeled protein maintains its capacity for binding to its antigen. The coupling is highly efficient when compared with other methods. The coupling of DTPA to proteins for radiolabeling has several other advantages that are not shared by methods in which radioiodine is used as the label. Specifically, the coupled antibody can be purified from the products of hydrolysis before the addition of radioactivity, thereby avoiding the need to handle radioactive samples during purification. Furthermore, the coupled and purified samples can be stored and radiolabeled only when required. The labeling by chelation with ^{111}In is extremely rapid and is, in effect, completed upon mixing of the activity and coupled protein solutions. Since a large number of metals form strong chelates with DTPA, they probably also form strong chelates with DTPA-coupled proteins. Thus, a variety of metallic radionuclides with different detection properties, radiation properties, and half-lives may be considered. We believe that the cyclic anhydride labeling method offers an attractive alternative to radioiodination for the radiolabeling of antibodies and other proteins.

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8. A Waters Associates Inc. (Milford, Mass.) HPLC was used in this study with I250 and I125 protein columns operated in series for improved resolution and with both ultraviolet detection (280 nm) and radioactivity detection. The eluant consisted of 2.5 percent triethylamine, 0.1*M* Na_2SO_4, *p*H 4.5.
9. The monoclonal antibody to PAP (an IgG 2a) was kindly supplied by New England Nuclear. The monoclonal antibody to CEA (an IgG 1k) and purified CEA were kindly supplied by Hoffmann–La Roche, Inc.
10. The animal tumor models used in this study, that is, the "tumored ear assay" and the "subrenal capsule assay," were originally developed by A. E. Bogden, Mason Research Institute, Worcester, Mass., for the rapid in vivo screening of chemotherapy drugs against a panel of human tumors transplantable in the athymic nude mouse. The cell line used in this study (designated CX-1) has previously been shown by radioimmunoassay of mouse plasma samples to excrete CEA in the range of 6 to 67 ng/ml, depending on tumor size, when implanted in the subrenal capsule. Implantation in the renal capsule is accomplished by withdrawing the right kidney of an anesthetized animal, inserting a trocar through a small slit in the capsule, and implanting 1-mm fragments of tumor under the capsule. The kidney is then returned, the body wall closed, and the animal permitted to recover. The pinnal implant is essentially a subcutaneous graft. A 2-mm tumor fragment is deposited in the pinna of an anesthetized animal by guiding the trocar along the pinnal ridge from an insertion site in the vicinity of the opposite shoulder.
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12. Radioiodinations, by the chloramine-T method, were performed by New England Nuclear. Viability was established for the labeled antibody to PAP by immunoradioactivity assay.
13. D. J. Hnatowich, unpublished results.
14. We thank A. E. Bogden, S. Speropoulos, and P. Hughes for help in the biodistribution studies and D. Haagensen for helpful discussions. Supported in part by NIH grants 1 RO1 CA26968 and 1 RO1 GM26780. Part of this work will be submitted by W.W.L. in partial fulfillment of the requirement for the Ph.D. degree at Northeastern University.

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Metastable Species of Hemoglobin: Room Temperature Transients and Cryogenically Trapped Intermediates

Abstract. *Resonance Raman spectra of photolyzed carbonmonoxyhemoglobin obtained with 10-nanosecond pulses are compared with the spectra of photolyzed carbonmonoxyhemoglobin stabilized at 80 K. In comparing the deoxy with the photodissociated species, the changes in the Raman spectra are the same for these two experimental regimes. These results show that at ambient and cryogenic temperatures the heme pocket in liganded hemoglobin is significantly different from that of deoxyhemoglobin. It is concluded that measurements of the properties of intermediate species from photodissociated hemoglobin stabilized at low temperatures can be used to probe the short-lived metastable forms of hemoglobin present after photodissociation under biologically relevant solution conditions.*

Binding of small molecular ligands to hemoglobin (Hb) brings about extensive changes in the structure of the heme macrocycle and the relation between the protein subunits that surround it. These changes can be studied by photodissociating a ligand and examining the resulting metastable species by time-resolved spectroscopy (*1*) or by steady-state spectroscopic examination of the metastable species trapped at cryogenic temperatures (*2–8*). However, the physiological relevance of the low-temperature studies has been an open question. We find that the low-frequency resonance Raman spectra of photolytic transients of carbonmonoxyhemoglobin (COHb) examined 10 nsec after photolysis at room temperature and those trapped at 80 K for long periods of time are equivalent (both deviate in the same ways from deoxyhemoglobin examined under the same conditions). Thus low-temperature measurements of photodissociated Hb provide a meaningful probe for the study of the dynamic character of the ligand-heme-globin interactions within the protein.

Reversible binding of small diatomic ligands such as O_2, CO, and NO to the heme in Hb is regulated by the surrounding protein configuration, but the specific interactions responsible for this regulation are not known. Indeed, it has not yet been determined whether the critical interactions occur near the ligand binding site (*9*) or throughout the protein (*10*). However, in metastable species generated immediately after binding or release of ligands, energetically important disturbances may be manifested, at least transiently, at the interface between the binding site and the surrounding protein. Under physiological conditions these metastable species persist on time scales ranging from picoseconds to milliseconds and are inaccessible to conventional steady-state spectroscopic techniques.

The relation between the two methods used to isolate and examine the transient species (Hb*) involved in ligand binding—time-resolved spectra of transients (*1*) or trapping and examination of metastable species at low temperature (*2–8*)—has been unexplored. Time-resolved studies of transients have been limited to optical absorption (*11–14*) and resonance Raman spectroscopy (*1*), while the low-temperature studies have primarily centered on electron paramagnetic reso-