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Synthesis of 1-(*p*-Isothiocyanatobenzyl) Derivatives of DTPA and EDTA. Antibody Labeling and Tumor-Imaging Studies

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To investigate the ^{111}In labeling of tumor-localizing monoclonal antibodies (MoAb), we have synthesized the chelate 1-(*p*-isothiocyanatobenzyl)diethylenetriaminepentaacetic acid (*p*-SCN-Bz-DPTA) (**1**) and its EDTA analogue (**2**). By the use of a MoAb (B72.3) specific for a high molecular weight antigen (TAG-72) on cells of a colorectal carcinoma grown in nude mice, optimal chemical conditions for MoAb conjugation of those ligands and of the dicyclic and isobutylcarboxy carbonic anhydrides of DTPA and subsequent ^{111}In labeling were determined. All conjugates were shown by a competitive binding assay to retain their specificity and activity in vitro when less than one ligand is protein coupled both prior to and after ^{111}In labeling. Chemical methods for purification of the MoAb were systematically investigated by injection of purified immunoprotein into athymic mice bearing LS-174T tumors that express the TAG-72 antigen. Tissue distribution studies revealed that simple addition of EDTA to labeled immunoglobulins was ineffective at complexing indium not linked to protein by chelates. Similarly, gel chromatography (Sephadex G-50) was not sufficient; rather, size exclusion HPLC had to be employed to remove unreacted ^{111}In and aggregated antibody. To compare the relative utility of the four chelates for ^{111}In diagnostic radioimmunoimaging, scintigraphic images of tumor-bearing mice were obtained and evaluated along with tissue distributions. Results showed that clear images of these solid tissue tumors free of extraneous radiation could be obtained only by using *p*-SCN-Bz-DTPA purified by HPLC. Methods developed are now being employed in clinical trials for diagnosis of human colorectal cancer.

Introduction

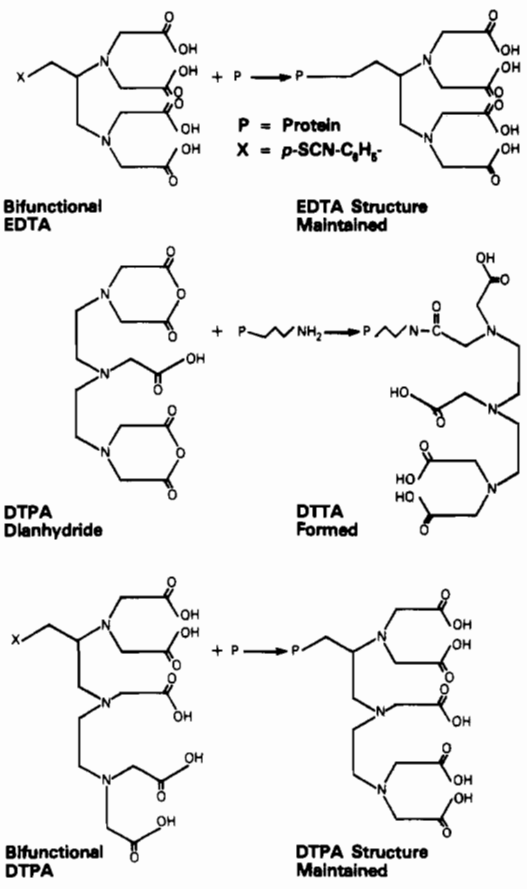
Current interest in irreversibly linking radioactive metal complexes to tumor-seeking immunoglobulins derives from observations that indium-111-labeled monoclonal antibody (MoAb), when injected in vivo, gives rise to scintigraphic images of malignant tissues.[1,2] Early MoAb-imaging studies[3-5] made use of iodine isotopes, but the possibility of in vivo degradation of carbon–iodine bonds[6] and the less-than-ideal emission characteristics and physical half-lives[7] of iodine radionuclides[2,7,8] make these radioisotopes marginally useful. Several reports[9-11] of experimental radioimmunodetection of human tumors with complexed metallic radionuclides have appeared in recent years, but before the technique can be of clinical use, studies are required to achieve sharper distinction between tumor and normal tissues.[12]

The various factors that determine tumor to background ratios in MoAb generated images are the radiolabeling chemistry, the inherent immunoreactivity of the MoAb, and the biological fate of labeled immunoglobulin. Biological and immunological issues have been reviewed and will not be discussed.[12-14]

Of particular importance is the choice of chelate. Chemically modified derivatives of diethylenetriaminepentaacetic acid (DTPA),[15-18] ethylenediaminetetraacetic acid (EDTA)[2,19-25] and the cryptands[26-28] are available. Covalent linkage to protein is accomplished by acylation with activated carbonyls, aromatic diazonium coupling, or bromoacetyl alkylation. These coupling methods are rather inefficient.[2,23,29,30] Extensive purification must be performed before labeling with metal radionuclides. A disadvantage of the two most commonly employed DTPA derivatives, the cyclic dianhydride (CA-DTPA)[18] and the mixed anhydride DTPA–isobutylcarboxy carbonic anhydride (MA-DTPA)[15], is that one ligand carboxylate metal binding site is occupied in an acid amide bond to form a protein-linked diethylenetriaminetetracetic acid (DTTA) (Chart I). One DTTA ligand is known to be lose indium faster in vivo than it is lost from DTPA.[30]

We therefore set out to find a chemically modified ligand that would react rapidly and efficiently with antibody, yet retain the metal for a time that is long compared to half-lives (1 h–3 days) of radionuclides thought useful for imaging or therapy.[7] Consideration was first given to the mode of protein linkage. Use of the isothiocyanate group for protein labeling has been extensively employed to efficiently couple fluorescence probes,[31] boron clusters,[32] or spin labels.[33] Moreover, isothiocyanates are very mild reagents, easily obtained from parent aliphatic or aromatic amines. The amine precursor of **2** was used by this group to obtain

Chart I. Methods for Covalently Linking Chelates to Protein



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the corresponding diazonium salt[1,2] and has, as well, been discussed elsewhere.[22,24] One report is an abstract,[24] while the other[22] outlines a synthesis, with scanty characterization, that generates a mixture of ortho and para isomers.[22,25] Additionally, we attempted to prepare 1-(*p*-(bromoacetamido)benzyl)ethylenediaminetetraacetic acid and -diethylenetriaminepentaacetic acid by the literature method[21,22] but found that both N-alkylated and *N*-acetyl products were obtained and could be separated only by high performance liquid chromatography (HPLC).

Among the radiometals of use for scintigraphy, ^{111}In is often selected. Its 68-h half-life allows sufficient time for tumor targeting and clearance of background immunoglobulin. Indium EDTA and DTPA complexes exhibit high stability constants and are inert to dissociation in water at plasma pH 7.2. Kinetics of formation and dissociation have been examined.[34,35]

Monoclonal antibody B72.3 was generated against a carcinoma metastasis and binds with high specificity to tumor-associated glycoprotein (TAG-72) found in human colon and breast cancer. Tissue section studies showed that TAG-72 is found in over 80% of colon carcinoma tested,[36-39] but no reactivity of B72.3 was found to normal adult tissue types. An animal model tumor bearing the TAG-72 antigen has been employed for ^{131}I scintigraphy.[40,41] Thus the B72.3–TAG-72 antibody–antigen pair is a clinically relevant model system with which to study in vitro and in vivo effects of metal chelate linkage and to investigate use of ^{111}In-labeled antibody for tumor imaging.

Herein, we show that the chemistry of MoAb labeling is a primary factor in determining tumor localization. The choice of chelate, the protocol for linkage to protein, and the method and extent of chemical purification of chemically modified MoAb are seen to affect the clarity of scintigraphic images of human tumors grown in immunosuppressed (athymic) mice. We now report detailed and efficient syntheses for **1**, **2**, their amino precursors, and some corresponding derivatives. Careful attention to the radiolabeling chemistry has led to marked improvement in tumor-to-organ isotope uptake ratios particularly in the liver and spleen. While intelligible visualizations of carcinoma can be seen for all chelates used in this study, only the new DTPA ligand provided tumor images virtually free of extraneous background.

Experimental Section

Materials and Methods. *p*-Nitrophenylalanine (Aldrich) was used without further purification after verification by NMR. Diborane (1 M BH_3·THF) solution was used as received (Alfa). All solvents were dried as needed. Stock solutions of all buffers used in this study were prepared by mixing appropriate volumes of saline solutions of acid and base buffer forms in concentrations 10 times those desired. Metal depletion of stock saline buffer solutions was accomplished by passage over a 1.5 × 8 cm column of Chelex 100 resin, 100–200 mesh (Bio-Rad), which had been preequilibrated with buffer. Citric acid, sodium citrate, and mono- and disodium phosphate were purchased from Mallinckrodt (AR grade), and HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid), MES (2-morpholinoethanesulfonic acid), and their salts were Ultrol grade purchased from Calbiochem Corp. [^{14}C]DTPA was purchased from DuPont/NEN. Bromo[$^{14}C_2$]acetic acid was obtained from Amersham Corp. Thin-layer chromatography (TLC) was performed on silica-60 plates purchased from EM Reagents. Column chromatography of ligands was performed by using Florisil (Sigma Chemical Co.). Ion exchange resins were obtained from Bio-Rad.

Proton NMR spectra were obtained by using either a Varian 220-MHz or a Nicolet NTC-500 instrument. Chemical shifts are reported in ppm on the δ scale relative to TSP (D_2O) or Me_4Si ([$^2H_6$]Me_2SO). All coupling constants (J values) are reported in hertz. Chemical ionization mass spectra (CI-MS) were measured with a Finnegan 3000 instrument. Electron-impact mass spectra (EI-MS) were recorded on a LKB 9000 instrument. Chemical analyses were performed at Galbraith Laboratories or at the Analytical Services Laboratory, NIADDK, NIH. High-performance liquid chromatography (HPLC) was performed with a Beckman Model 746 system controlled by a Beckman 450 data system. Analytical HPLC was performed by using a 4.56 mm × 15 cm, 5 μm silica ODS C_{18} column, which was eluted with a linear gradient of pure aq. 0.05 M triethylammonium acetate to 100% methanol at a flow rate of 1 mL/min for 25 min.

Methyl *p*-Nitrophenylalanine Hydrochloride (4)[41] (Me-*p*-NO_2-Phe). *p*-Nitrophenylalanine (10.0 g, 47.6 mmol) was treated with methanol (200 mL) saturated with HCl(g) and left to stir at room temperature for 18 h. The solution was evaporated to near dryness and the precipitated product collected in a Büchner funnel. After the product was dried under vacuum at 50 °C, the yield was 10.97 g (88.3%). A TLC of the free amino ester developed in $CHCl_3$–MeOH (4:1) revealed an R_f value of 0.85–0.88.

^{1}H NMR (220 MHz, D_2O, pH 1.5): δ 8.20 (d, 2 H, J = 10.0), 7.53 (d, 2 H, J = 10.0), 4.55 (t, 1 H, J = 5.00), 3.84 (s, 3 H), 3.43 (m, 2 H).

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p-Nitrophenylalanine Amide (5)[42] (p-NO_2-Phe-amide). A slurry of **4** (10.97 g, 42.1 mmol) in dry methanol (5 mL) was treated with triethylamine (6.45 mL). Anhydrous ether (200 mL) was added, and the solution was cooled at −10 °C for 1 h. The triethylamine hydrochloride was filtered off and the filtrate concentrated to a limpid yellow oil. The oil was added to dry methanol (250 mL) previously saturated with NH_3(g) at −10 °C, tightly stoppered and left at −10 °C for 42 h. The precipitated product was collected and dried under vacuum. The filtrate was found to be a pure solution of the product by TLC ($CHCl_3$–MeOH, 4:1). The filtrate was therefore stripped to dryness and the remaining solid combined with the precipitate (8.12 g, 92.3%); mp 160–162 °C.

^{1}H NMR (220 MHz, [$^2H_6$]Me_2SO): δ 8.07 (d, 2 H, *J* = 8.00), 7.5 (d, 2 H, *J* = 8.00), 7.41 (s, 1 H), 7.00 (s, 1 H), 3.50 (t, 1 H, *J* = 3.00), 3.09 (dd, 1 H, *J* = 8.00, 3.00), 2.81 (dd, 1 H, *J* = 8.00, 3.00).

1-(p-Nitrobenzyl)ethylenediamine Dihydrochloride (6)[43] (p-NO_2-Bz-en). A 1-L three-neck round-bottom flask was fitted with a reflux condenser, septum, argon inlet, and bubbler exit and then flame-dried. **5** (8.12 g, 38.9 mmol) was washed into the reaction flask with dry THF (150 mL) and cooled to −10 °C. Next, a 1 M BH_3·THF solution (200 mL) was added with a syringe. The reaction solution was stirred for 1 h at −10 °C and then raised to a gentle reflux for 18 h, after which it was cooled to −10 °C and dry methanol (25 mL) was injected. The reaction was brought to room temperature after which the solvent was removed. Methanol (25 mL) again was added and evaporated to near dryness. The residue was taken up in dry ethanol (100 mL), saturated with HCl(g), and heated to reflux for 2 h. The reaction was then tightly stoppered and left at 0 °C for 18 h. The precipitated product (8.86 g, 85.1%) was collected and dried under vacuum.

^{1}H NMR (500 MHz, D_2O, pH 1.0): δ 8.234 (d, 2 H, *J* = 8.00), 7.634 (d, 2 H, *J* = 8.00), 4.125 (m 1 H), 3.543 (dd, 1 H, *J* = 15.00, 7.50), 3.467 (dd, 1 H, *J* = 15.00, 7.50), 3.391 (dd, 1 H, *J* = 14.00, 7.50), 3.260 (dd, 1 H, *J* v 14.00, 7.50). ^{1}H NMR (500 MHz, D_2O, pH 11.5): δ 8.046 (d, 2 H, *J* = 8.50), 7.423 (d, 2 H, *J* = 8.50), 3.061 (m, 1 H), 2.926 (dd, 1 H, *J* = 13.25, 5.25), 2.730 (dd, 1 H, *J* = 13.25, 4.50), 2.665 (dd, 1 H, *J* = 13.25, 5.25), 2.592 (dd, 1 H, *J* = 13.25, 7.50).

1-(p-Nitrobenzyl)ethylenediaminetetraacetic Acid (7)[22,23] (p-NO_2-Bz-EDTA). A 50-mL three-neck round-bottom flask was fitted with the addition tube from the Radiometer ABU 80 autoburette, a glass electrode, a stirring bar, and a stopper. The reaction flask was charged with **6** (2.0 g, 7.465 mmol) in H_2O (6mL) and heated in an oil bath to 45 °C. Bromoacetic acid (4.198 g, 30.2 mmol) was added in four equal portions over 4 h, while the reaction mixture was maintained at pH 10.8 by the controlled, dropwise addition of 7 M KOH. After the final addition, the reaction was allowed to continue for 18 h before filtration through a medium glass frit, acidification with concentrated HBr to pH 1.8, and evaporation to dryness were performed. A substantial amount of salt was removed by triturating with hot 100% formic acid followed by filtration on a medium frit. The filtrate was evaporated and the residue taken up in 1 M formic acid and loaded onto an ion-exchange column made up of a 2.6 × 30 cm bed of AG1-X8, 200–400 mesh anion-exchange resin, formate form. The column was jacketed and maintained at 40 °C by a water bath. The product was eluted from the column with a 1–7 M gradient of formic acid at a flow rate of 2 mL/min, monitoring at 370 nm, with a 6.5 mL fraction size. Fractions 155–206, which contained precipitated product, were collected by filtration, and the filtrate was combined with the eluant in fractions 135–237 and stripped to dryness (2.234 g, 70.0%). TLC of the product was performed in 1-butanol–H_2O–HOAc (4:1:1) with R_f 0.20 being found and in NH_4OAc–MeOH (1:1) with an R_f 0.80–0.83.

^{1}H NMR (500 MHz, D_2O, pH 4.5): δ 8.176 (d, 2 H, *J* = 8.00), 7.577 (d, 2 H, *J* = 8.00), 3.928 (d, 2 H, *J* = 17.0), 3.839 (q, 4 H, *J* = 17.0), 3.685 (d, 2 H, *J* = 17.0), 3.581 (t, 1H, *J* = 12.0), 3.388 (7, 2 H), 3.006 (t, 1 H, *J* = 10.0). ^{1}H NMR (500 MHz, D_2O, pH 11.5): δ 8.098 (d, 2 H, *J* = 7.50), 7.398 (d, 2 H, *J* = 7.50), 3.40–3.00 (m, 7 H), 2.888 (t, 1 H, *J* = 10.5), (d, 2 H, *J* = 16.0), 2.562 (t, 1 H, *J* = 13.0), 2.462 (t, 1 H, *J* = 11.5), (d, 1 H, *J* = 14.0). An EI-MS sample was prepared by reacting **7** with excess bis(trimethylsilyl)trifluoroacetamide in acetonitrile.

1-(p-Aminobenzyl)ethylenediaminetetraacetic Acid (8)[2,22,23] (p-NH_2-Bz-EDTA). A water-jacketed three-neck flask (50 mL) was charged with 10% Pd/C (43 mg), H_2O (5 mL), and a stirring bar. The center neck was attached to an atmospheric hydrogenation apparatus; one side neck was fitted with an injection valve, and the remaining neck firmly stoppered. The assembled hydrogenation apparatus was evacuated and flushed with hydrogen while the reaction flask was cooled to 4 °C. **7** (427 mg, 1 mmol) was dissolved in H_2O (10 mL), and 5 M NaOH was added to bring the pH to 10.0. The solution was injected into the reaction flask and hydrogen uptake monitored. After 67.5 mL of gas had been consumed, the flask was disconnected from the system and the contents filtered through a fine frit coated with Celite 535. The filtrate was acidified to pH 2.0 and stripped to near dryness, and the residual solution was loaded onto an ion exchange column, 2.6 × 30 cm, loaded with AG50W-X8, 200–400 mesh, H^+ form, and eluted with 0.5 M NH_4OH. The fractions containing product, as determined by TLC, were evaporated to a solid and dried under vacuum for 18 h (390 mg, 98.0%). The TLC of the product had R_f = 0.10 in 1-butanol–H_2O–HOAc (4:1:1).

^{1}H NMR (500 MHz, D_2O, pH 1.5): δ 7.468 (m, 4 H), 4.109 (d, 2 H, *J* = 17.5), 4.020 (d, 2 H, *J* = 17.0), 3.768 (d, 2 H, *J* = 17.5), 3.640 (d, 2 H, *J* = 15.0), 3.887 (m, 2 H), 3.193 (d, 1 H, *J* = 13.0), 2.798 nd, 1 H, *J* = 13.0), one proton obscured by doublet at 3.640. ^{1}H NMR (500 MHz, D_2O, pH 6.0): δ 7.173 (d, 2 H, *J* = 7.00), 6.959 (d, 2 H, *J* = 7.00), 3.832 (d, 2 H, *J* = 16.5), 3.699 (d, 2 H, *J* = 16.5), 3.624 (d, 2 H, *J* = 16.5), 3.568 (d, 2 H, *J* = 16.5), 3.346 (t, 1 H, *J* = 9.50), 3.233 (d, 1 H, *J* = 14.0), 3.131 (d, 1 H, *J* = 9.50), 2.679 (t, 1 H, *J* = 13.0), one proton obscured by doublet at 3.624. ^{1}H NMR (500 MHz, D_2O, pH 11.5): δ 7.033 (d, 2 H, *J* = 7.00), 6.805 (d, 2 H, *J* = 7.00), 3.40–3.00 (m, 4 H), 3.007 (d, 2 H, *J* = 16.5), 2.841 (d, 1 H, *J* = 13.0) 2.725 (t, 1 H, *J* = 12.0), 2.659 (d, 2 H, *J* = 16.5), 2.429 (t, 1 H, *J* = 12.0), 2.171 (d, 1 H, *J* = 13.0), 2.096 (t, 1 H, *J* = 12.0). An EI-MS sample was prepared by reacting **8** with excess bis(trimethylsilyl)trifluoroacetamide in acetonitrile.

1-(p-Isothiocyanatobenzyl)ethylenediaminetetraacetic Acid (2) (p-SCN-Bz-EDTA). Method A. The precursor **8** (0.33 mmol) was taken up in H_2O (10 mL) and stirred rapidly in a 10-mL round-bottom flask fitted with an addition funnel. The pH was adjusted to 8.5 with solid $NaHCO_3$, and thiophosgene (42 mg, 0.365 mmol) in $CHCl_3$ (10 mL) was added dropwise. Stirring was continued until the solution tested negative for amine by the fluorescamine method.[44] The aqueous layer was taken to dryness. Purification was done by column chromatography on a 1 × 30 cm Florisil column eluted with acetonitrile–H_2O (30:8). The product eluted from the column cleanly as determined by TLC and was stored in a desiccator in a freezer and repurified immediately prior to use.

The R_f of the product, determined by TLC, was 0.45 in acetonitrile–water (30:8) and 0.60 in ethanol–water–acetic acid (70:25:5). The IR spectra showed an absorption at 2100 cm^{-1} in Nujol.

^{1}H NMR (500 MHz, D_2O, pH 5.3): δ 7.273 (q, 4 H, *J* = 8.50), 3.471 (d, 1 H, *J* = 17.0), 3.313 (br s, 2 H), 3.118 (d, 1 H, *J* = 14.0), 3.05–2.70 (m, 6 H), 2.547 (t, 1 H, *J* = 13.5), 2.447 (t, 1 H, *J* = 13.0), 2.257 (d, 1 H, *J* = 14.0).

Method B. Compound **7** (402.0 mg, 0.941 mmol) was reduced as in the procedure for the preparation of **8** except that tetrabutylammonium hydroxide, 25% in methanol, was used instead of 5 M NaOH. Workup was identical, but the product was a thick dark reddish oil. This oil was taken up in H_2O (10 mL), and solid $NaHCO_3$ was added to raise the pH to 8.5. Thiophosgene, 162 mg (1.41 mmol), in $CHCl_3$ (10 mL) was then added dropwise with rapid stirring, which was continued until the reaction solution tested negative to fluorescamine.[44] The reaction mixture was poured into a separatory funnel, and the water layer was extracted twice with $CHCl_3$ (10 mL). The aqueous layer was evaporated to a solid. This procedure resulted in a >95% pure (by NMR) product in all ways identical with the purified material obtained in Method A.

Attempt To Prepare 1-(p-Bromoacetamidobenzyl)ethylenediaminetetraacetic Acid. Bromoacetyl bromide (0.053 mL) was reacted with the amine **8** (0.504 mmol) as described by De Riemer et al.[21,22] Contrary to reported observations, a solid was obtained immediately upon mixing of the reagents. The precipitate was collected and dried under vacuum and was shown by NMR to be a mixture of N-alkylated and N-acetylated products.

^{1}H NMR (220 MHz, D_2O, pH 4.5): δ 7.50–7.11 (m), 4.05 (s), 3.95–3.02 (m), 2.86–2.57 (m); integration confirmed a mixture of products.

***N*-(2-Aminoethyl)-*p*-nitrophenylalanine Amide (9).** **4** (4.4 g, 17.9 mmol) was treated with triethylamine (2.78 mL, 20.0 mmol) to generate the free amino ester. The free ester was taken up in methanol (5 mL) and added dropwise to ethylenediamine (35 mL) at room temperature with vigorous stirring. TLC revealed that the reaction was essentially completed in less than 1 h, and the reaction was allowed to stir for 18 h. The reaction flask was fitted with a vacuum takeoff adaptor and attached to a vacuum pump with a liquid-nitrogen trap in line. Removal of the solvent left a brownish solid, which was dried at 50 °C under vacuum for 6 h and left under vacuum for 24 h at room temperature (4.42 g, 87.5%). TLC of the product showed R_f = 0.10–0.12 in chloroform–methanol (4:1).

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(43) Brown, H. C.; Keim, P. *J. Org. Chem.* **1973**, *38*, 912–916.
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^{1}H NMR (220MHz, D_2O, pH 1.0): δ 8.23 (d, 2 H, J = 8.00), 7.54 (d, 2 H, J = 8.00), 4.36 (t, 1 H, J = 7.00), 3.62 (m, 1 H), 3.41 (m, 3 H), 3.12 (m, 2 H).

(*p*-Nitrobenzyl)diethylenetriamine Trihydrochloride (10) (*p*-NO_2-Bz-dien). **9** (5.38 g, 21.3 mmol) was reduced with 1 M BH_3·THF (125 mL) in a manner identical with that employed in the preparation of **6** (6.05 g, 82.3%).

^{1}H NMR (500 MHz, D_2O, pH 1.5): δ 8.285 (d, 2 H, J = 9.00), 7.605 (d, 2 H, J = 9.00), 4.118 (m, 1 H), 3.68–3.57 (m, 2 H), 3.538 (t, 2 H, J = 7.00), 3.458 (t, 2 H, J = 7.00), 3.378 (dd, 1 H, J = 12.5, 9.00), 3.213 (dd, 1 H, J = 12.5, 9.00).

Alternative Synthetic Pathway to 10 (for Verification Purposes). (*tert*-Butoxycarbonyl)-*p*-nitrophenylalanine (13)[45] (BOC-*p*-NO_2-Phe). *p*-Nitrophenylalanine (7.94 g, 37.8 mmol) was dissolved in 50% aqueous dioxane solution (60 mL) and triethylamine (7.9 mL, 56.7 mmol) added. BOC-ON (10.24 g, 41.6 mmol) (Aldrich Chemical Co.) was added and the solution stirred for 2 h. Ethyl acetate (100 mL) and H_2O (50 mL) were next added and the contents poured into a separatory funnel. The aqueous layer was retained and extracted twice with ethyl acetate (100 mL). The aqueous layer was cooled to 0 °C, and the pH was adjusted to 2.0 with 3 N HCl, whereupon a precipitate formed, which was collected and dried under vacuum. The filtrate was extracted with ethyl acetate twice (100 mL), dried over $MgSO_4$, and stripped to dryness. The two fractions proved to be identical and were combined (11.0 g, 94.0%). The melting point of the compound was 165 °C.

^{1}H NMR (220 MHz, [$^2H_6$]Me_2SO): δ 8.036 (d, 2 H, J = 8.00), 7.29 (d, 2 H, J = 8.00), 5.38 (d, 1 H, J = 8.00), 4.44 (m, 1 H), 3.25 (dd, 1 H, J = 13.0, 6.00), 3.05 (dd,1 H, J = 13.0), 6.00), 1.39 (s, 9 H).

1-(*p*-Nitrobenzyl)diethylenetriamine Trihydrochloride (10)[46] (*p*-NO_2-Bz-dien). Glycine methyl ester hydrochloride (1.79 g, 14.26 mmol) was dissolved in ethyl acetate (150 mL) and cooled to 0 °C. Triethylamine (1.44 g, 14.26 mmol), **13** (4.42 g, 14.26 mmol), and 1-hydroxybenzotriazole (1.69 g, 12.55 mmol) were added. DCC (3.231 g, 15.68 mmol) in ethyl acetate (10 mL) was added. The flask was stoppered with a drying tube, allowed to come to room temperature, and stirred for 48 h. Three drops of acetic acid were added, and after 10 min, the reaction mixture was filtered. The filtrate was retained and extracted in succession with 0.5 N HCl (3 × 50 mL), saturated NaCl (50 mL), 5% $NaHCO_3$ (3 × 50 mL), and again with saturated NaCl (50 mL). The wet organic phase was dried ($MgSO_4$) and evaporated to a solid, which was dried under vacuum for 18 h. The dipeptide **14** (5.40 g, 14.73 mmol) was hydrolyzed by allowing it to stand for 2 h in dioxane (60 mL) into which had been poured 1 M KOH (40 mL). The dioxane was evaporated and the aqueous solution acidified with citric acid to pH 5.0. The aqueous solution was extracted with ethyl acetate (3 × 100 mL). The combined organic extracts were dried ($MgSO_4$) and evaporated to a solid, which was dried under vacuum for 18 h. The acid **15** (4.75 g, 12.9 mmol) was dissolved in ethyl acetate (450 mL), and DMF was added dropwise to effect a clear solution. *N*-Hydroxysuccinimide (1.496 g, 13.0 mmol) was added, followed by DCC (2.68 g, 13.0 mmol) dissolved in ethyl acetate (10 mL). The solution was allowed to stir for 12 h, after which it was filtered and returned to the reaction flask. Dioxane (10 mL) saturated with NH_3(g) was added dropwise, and an immediate precipitate formed. The solution was stirred an additional 2 h. Afterward, the precipitate was collected and dried under vacuum for 18 h. The dipeptide amide **16** (5.1 g, 13.1 mmol) was deprotected by being stirred for 4 h in dioxane (100 mL) to which had been added 3 N HCl (200 mL). The solution was evaporated to dryness, taken up in methanol (25 mL), and again evaporated to a solid, which was dried under vacuum for 18 h. The deblocked **17** (2.9 g, 9.52 mmol) was reduced by 1 H BH_3·THF (60 mL) in a manner identical with the preparation of **6**. The isolated solid (34.2% yield overall) was in all respects identical with that from the reduction of **10**.

1-(*p*-Nitrobenzyl)diethylenetriaminepentaacetic Acid (11)[47] (*p*-NO_2-Bz-DTA). Bromoacetic acid (2.0 g, 14.4 mmol) was dissolved in toluene (2 mL) and cooled to 0 °C. KOH, 7 M (5.47 mL), was added slowly, followed by the addition of **10** (1.0 g, 2.6 mmol). The reaction was allowed to come to room temperature and to stir for 24 h. Bromoacetic acid (4.0 g, 28.8 mmol) was added again, followed by 7 M KOH (8.22 mL), and the reaction solution was allowed to stir for 72 h at room temperature. The solution was acidified to pH 1.5 with concentrated HBr and extracted with ether (3 × 100 mL). The aqueous solution was evaporated to a solid and loaded onto an ion-exchange column of AG50W-X8, 200–400 mesh, H^+ form, 2.6 × 30 cm, and washed with H_2O to remove the unreacted materials, hydrolysis products, and salts. The crude product was eluted with 2 N aqueous NH_3. The crude product was further purified by HPLC using a 10 × 250 mm, C_{18} reverse-phase column with a gradient of aqueous 0.05 M triethylammonium acetate to 100% methanol at a flow rate of 3 mL/min. The product had a retention time of 9.1 min. The combined fractions from the HPLC were rechromatographed on an AG50W-X8 column identical with the first column to remove the triethylammonium acetate buffer. The product was collected and the solvent evaporated to give a solid. (485 mg, 35.3%).

^{1}H NMR (220 MHz, D_2O, pH 12.0): δ 8.20 (d, 2 H, J = 8.00), 7.48 (d, 2 H, J = 8.00), 3.57 (q), 3.42 (d), 3.32 (t), 3.07 (m), 2.90 (t), 2.695 (m). The alkyl region is described with only the major signals noted as there are many complex patterns buried beneath these clearer signals. The area does integrate properly for 19 protons vs. the four aromatic protons. An EI-MS sample was prepared by reacting the product with excess bis(trimethylsilyl)trifluoroacetamide in acetonitrile.

1-(*p*-Aminobenzyl)diethylenetriaminepentaacetic Acid (12) (*p*-NH_2-Bz-DTPA). **11** (454 mg, 0.86 mmol) was reduced by Pd/C (50 mg) by using a procedure identical with that used for the production of **8**. After the reaction solution was filtered through a fine frit with Celite 535, the solvent was removed and the solid dried under vacuum for 18 h.

^{1}H NMR (220 MHz, D_2O, pH 1.0): δ 7.45 (q, 4 H), 4.01 (d), 3.9–3.64 (m), 3.6–3.09 (m), 2.87 (m). As with **11**, the alkyl region is described with only the major features noted due to the complexity of the spectrum.

1-(*p*-Isothiocyanatobenzyl)diethylenetriaminepentaacetic Acid (1) (*p*-SCN-Bz-DTPA). The amine **12** (400 mg, 0.758 mmol) was converted to the crude product by reaction with thiophosgene (0.61 mL, 0.795 mmol) by using the same procedure employed to generate **2**. The crude product was purified by column chromatography on a 1 × 30 cm Florisil column eluted with acetonitrile–H_2O (30:8). The product eluted from the column cleanly and was freeze-dried and stored in a vacuum desiccator.

The R_f of the product on a silica-60 TLC plate developed with acetonitrile–H_2O (30:8) was 0.20. The IR spectra showed an absorption at 2100 cm^{-1}.

^{1}H NMR (220 MHz, H_2O, pH 6.5): δ 7.38 (s, 4 H); the alkyl region (2.5–3.86) showed only a poorly resolved signal at this pH, which integrated for 19 protons vs. the four aromatic protons.

Antibody Preparation. Monoclonal antibody B72.3 was generated by using a membrane-enriched fraction of cells from a mammary carcinoma metastasis as described elsewhere.[36] Antibody employed in this study was purified by using previously reported methods.[40]

Coupling of Chelates to Antibody B72.3. MA-DTPA.[15] A modification of the method of Krejcarek and Tucker was used to link MA-DTPA to B72.3 with [^{14}C]DTPA being employed to quantitate the number of chelates bonded to antibody. To perform the reaction at an initial 100:1 chelate:antibody ratio, DTPA (0.2 mmol) was dissolved in water by addition of triethylamine (1.38 mmol) and lyophylized. The solid formed was taken up in 1 mL of dry CH_3CN at 4 °C and treated with isobutylchloroformate (0.27 mmol) for 30 min and centrifuged, and an aliquot (12.2 μL) was reacted at 4 °C for 1.5 h with B72.3 (1 mL, 3.8 mg/mL in 50 mM HEPES, pH 8.5).

Sequential dialyses in metal-free buffers were employed to purify protein from unreacted ligand. The first dialysis solution was 50 mM citrate, pH 5.5, 0.15 M NaCl, and 0.1 mM ascorbic acid; the two subsequent dialyses were the same except for omission of the ascorbate. Washed Chelex 100 ion-exchange resin (Bio-Rad) was added to all citrate dialyses. The final dialysis solution was 0.02 M MES, pH 5.9, and 0.15 M NaCl and did not contain Chelex 100 resin. Quantification of chelate linked to B72.3 by scintillation counting, as described below, and a colorimetric protein assay (Bio-Rad) showed a protein concentration of 2.7 mg/mL with about 0.8 chelate/antibody molecule.

CA-DTPA. ^{14}C-DTPA dianhydride was prepared by the method of Eckelman.[48] For reaction with B72.3 at an initial 2:1 mole ratio of chelate to antibody, a solution of CA-DTPA (3.3 mg in 706.6 μL of dry Me_2SO, 13 mM) was prepared and a 3.9 mL aliquot reacted at 25 °C for 1 h with B72.3 (1 mL, 3.8 mg/mL in 50 mM Hepes, pH 8.5). Purification of protein from unreacted ligand was performed by sequential dialyses as described above. Radiometric quantitation showed 0.9 chelates per antibody after dialyses.

Isothiocyanate Chelates 1 and 2. ^{14}C-labeled isothiocyanate chelates **1** and **2** were prepared as described above by use of $Br^{14}CH_2COOH$. To react the isothiocyanates with B72.3 at an initial 3:1 ratio of chelate to antibody, a 5.0 mM aqueous solution of chelate was prepared immedi-

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(46) Bara, Y. A.; Friedrich, A.; Hehlein, W.; Kessler, H.; Kandor, P.; Molter, M.; Veith, H.-J. *Chem. Ber.* **1978**, *111*, 1029–1044.
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(48) Eckelman, W. C.; Karesh, S. M.; Reba, R. C. *J. Pharm. Sci.* **1975**, *64*, 704–706.

ately before use by dissolution of weighed solid and 10 μL was added to the protein solution (0.5 mL, 5.0 mg/mL) in 50 mM Hepes, pH 8.5). The 16 μL of 1.5 M aqueous triethylamine, pH 8.0, was added as a general base catalyst. After 2 h reaction time, MoAb was purified of unreacted chelate by dialysis. Standard scintillation counting procedures revealed an average of 1.4 chelates linked to an antibody.

Determination of Average Number of Chelates Linked to an Antibody Molecule. Quantification of the average chelate:antibody ratio was accomplished by scintillation counting and measurement of protein concentration of the product of the coupling reactions described above. The methodology employed will be described here only for the linkage of the substituted EDTA **2**. To introduce the label, 3.0 mCi of Br-$^{14}CH_2COOH$ was added to the amine alkylation step of the preparation of the *p*-nitrobenzyl–EDTA complex **7**. This compound was isolated as the analytically pure solid, and a weighed amount was scintillation counted with appropriate quench and efficiency correction and compared to a ^{14}C standard (Amersham) to determine a specific activity of 0.89 μCi/μmol for labeled **7**, which was then converted to the isothiocyanate and linked to B72.3 as described above. Protein concentration was measured by either the Bio-Rad assay or by determination of absorbance at 280 nm. To determine ligand concentration in the protein solution, a measured volume was counted and corrected for quench and efficiency, to determine the activity present in μCi units, and divided by the specific activity and volume. This ratio of ligand concentration to protein concentration provided the desired average chelate:antibody ratio.

^{111}In Labeling. To a solution of 60 μL of ^{111}In (3 mCi, 0.05 M HCl, Dupont-NEN) was added 26.4 μL of 2 M HCl. After 5 min, the solution was neutralized to pH 4.2 with 26 μL of 1 M sodium acetate. Then 200 μL of B72.3 solution (3 mg/mL, 20 mM MES, 0.15 M NaCl, pH 6.0) was immediately added and allowed to react for 30 min before purification as described below. Protein concentration was then measured and ^{111}In activity determined by use of a Capintec dose calibrator. Specific activity ranged from 3 to 5 mCi/mg, but higher values (30 mCi/mg) were obtained by use of more ^{111}In and less protein while reaction solution volumes were kept as small as possible.

Purification of ^{111}In-Labeled B72.3. Four protocols were employed in order to test the importance of purification methods for obtaining good tissue distributions and tumor images with ^{111}In-labeled B72.3. All four were employed to purify B72.3 labeled with ^{111}In and chelate **1** to do tissue distribution studies.

EDTA "Chase" Method. B72.3 labeled with ^{111}In by using chelate **1** as described above was purified according to the general method first suggested as Goodwin et al.[49] Thereby, the ^{111}In-labeling solution described above was added to 1 mL of a solution of 0.1 mM EDTA, 0.1 M citrate, and pH 5 and allowed to sit for 1 h prior to injection into mice. Samples were diluted with PBS (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.1) just before use.

Gel Column Chromatography. Sephadex G-50 (fine) was swollen overnight in the PBS buffer used for protein elution. A column 1 cm × 8 cm was prepared and the unpurified ^{111}In B72.3 labeling solution was chromatographed with collection of 0.5 mL aliquots. Tubes 5 and 6 usually contained about 2.1 mCi of labeled protein and were pooled for use. About 80% of the protein was recovered.

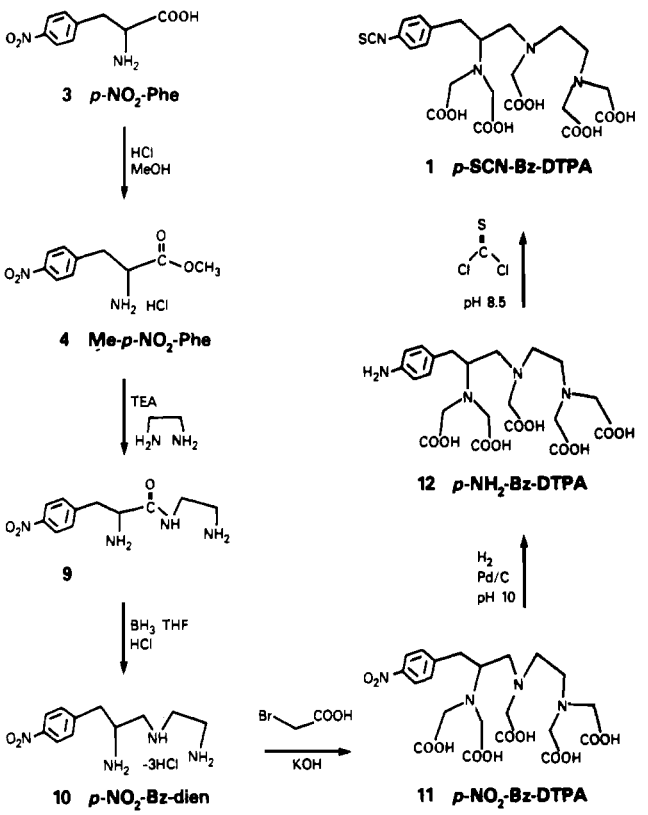
HPLC Purification. Protein B72.3 labeled with ^{111}In was injected into an LKB Model 700 HPLC equipped with a 7.5 mm × 30 cm TSK 3000 column, UV and radiometric detectors, dual pen recorder, and Super-rac fraction collector. Protein aggregates cleanly separated at a retention time of 6.5 min at a flow rate of 1 mL/min of PBS while labeled IgG appeared at 9.5 min. Aggregate peaks usually accounted for about 10% of both protein and Indium-111 label. Specific activity was usually about 3–6 mCi/mg of recovered protein (80%), which was collected and pooled from three 0.5-mL fractions.

Gel Column plus HPLC Purification. The pooled eluant of the gel column separation described above was further purified by injection into the HPLC of 0.5 mL of protein solution, elution with PBS, and collection of protein as described above. Both aggregate and IgG were detected. Usually about 30% of activity was lost in this final purification for net recovery of 65% of MoAb.

After purification, each antibody preparation used for imaging or tissue distribution studies was measured for retention of immunoreactivity by solid-phase RIA against human cell extracts.[50]

Animal Studies. Tissue distribution studies were conducted in approximately 7-week-old female mice, which had been inoculated subcutaneously in the flank 2 weeks previous with 0.4–1 million cells of the LS-174T human colon cancer line (positive for TAG-72) or the control

Chart II. Synthetic Pathway to 1-(*p*-Isothiocyanatobenzyl)diethylenetriaminepentaacetic Acid



A375 human melanoma line (negative for TAG-72). Tumors of 0.5–1.0-cm diameter had formed. Forty animals were intravenously (iv) injected with about 10 μCi of ^{111}In-labeled B72.3 and then sacrificed at suitable time points for necropsy studies in which tumor, blood, and five solid organs were weighed and γ-counted after exsanguination. The activity as a percentage of total radioactive dose injected per gram tissue was then calculated. Ratios of activity in tumor to that in other organs, i.e. tumor:liver (T:L) and tumor:kidney (T:K), were then formed from this data. A complete discussion of biological results will be presented elsewhere.[51]

For imaging studies to compare the utility of the four chelates examined, four animals were each injected iv with 10–20 μCi of ^{111}In linked to B72.3 by the various ligands and purified by both column chromatography and HPLC. Sequential images were obtained daily over 7 days by use of a protocol that has been detailed elsewhere.[40]

Results

A general method for synthesis of a 1-benzyl-substituted DTPA or EDTA may be summarized as a two-step process consisting of the generation of substituted ethylenediamines or diethylenetriamines followed by alkylation of the amines to form the corresponding polyacetic acid (Chart II). With this accomplished, a functional group of the benzyl substitution may be converted to a reactive moiety useful for protein coupling.

Synthesis of 1-(*p*-Isothiocyanatobenzyl)ethylenediaminetetraacetic Acid (1). To prepare a benzyl–EDTA derivative, Yeh et al.[19] first proposed that a substituted amino acid could be readily converted to an ethylenediamine in good yield and with high purity followed by alkylation to make the polyacetate. Since an isothiocyanate was the synthetic objective, the substituent we chose on the amino acid was a *p*-nitroaryl group, which would be rel-

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atively inert yet serve as a ready precursor to an amine that could subsequently be converted to an isothiocyanate.

Commercially available *p*-nitrophenylalanine was esterified in good yield and the resultant ester hydrochloride **4** was liberated with a tertiary amine and treated with saturated ammonia solution to generate the *p*-NO_2PheAla-amide **5** in good yield. Reduction of **5** to *p*-NO_2-Bz-en (**6**) was accomplished by using excess diborane followed by an acidic ethanolic reflux. This facile workup was found essential to avoid poor yields of a literature method[19] for breakup of boron ethylenediamine adducts that were not efficiently removed from *p*-NO_2-Bz-en with a simple aqueous reaction or by refluxing in acidic methanol.

The tetraacetic acid portion of *p*-NO_2-Bz-EDTA (**7**) was formed by a standard amine alkylation technique employing excess bromoacetic acid and aqueous KOH. Purification of **7** was accomplished by ion-exchange chromatography, which separated the unreacted starting materials and a principal side product, an ethylenediaminetriacetic acid, from the desired *p*-NO_2-Bz-EDTA. Reduction of the nitro group with Pd/C in basic media unmasked the amino group of *p*-NH_2-Bz-EDTA (**8**) for conversion to an isothocyanate.

Treatment of *p*-NH_2-Bz-EDTA with thiophosgene was found to generate *p*-SCN-Bz-EDTA (**2**) with approximately 80% purity as determined by NMR. Column chromatography on Florisil proved to be the most gentle and efficient method of purifying a large quantity of the desired isothiocyanate.

In an attempt to generate *p*-SCN-Bz-EDTA in higher initial purity, reduction of the precursor amine was carried out in an essentially identical manner but by using $(Bu)_4NOH$ as base. The resulting oil was then treated with thiophosgene to produce *p*-SCN-Bz-EDTA in >95% purity, as determined by NMR.

Synthesis of the *p*-(bromoacetamido)benzyl derivative by reaction of bromoactetyl bromide with the amine **8** was not possible in our hands, and in fact, we find the product of the reported reaction[21,22] to be only in a minor part the desired compound with mostly the N-alkylated material seen. Examination of the ^1H NMR spectrum revealed a complex multiplet of aryl signals, indicative of the mixture, and, most importantly, a new, dominant singlet at 3.7 ppm, which clearly results from the additional nitriloacetate methylene generated by N-alkylation at the methylene carbon of bromoacetyl bromide. A singlet observed at 4.05 ppm, the appropriate position for the N-acetylated product at pH 4.5, was only ca. 30% as intense as expected by integration vs. aromatic protons.

1-(*p*-Isothiocyanatobenzyl)diethylenetriaminepentaacetic Acid (2). To prepare the DTPA analogue of *p*-SCN-Bz-EDTA, the synthesis was modified to include substitution at the amide to introduce the additional carbon framework, while the remainder of the synthesis was unchanged (Chart II). To confirm structure, an alternative synthesis was devised and carried out by using classical peptide chemistry. The amine of *p*-nitrophenylalanine was first blocked as carbamate **13**. Next, the *tert*-butyloxycarbonyl (BOC) acid, BOC-*p*-NO_2-Phe (**13**), was coupled to methyl glycine by using dicyclohexylcarbodiimide (DCC) to complete the carbon framework of diethylenetriamine. The Me-BOC-*p*-NO_2-PheGly (**14**) was hydrolyzed to BOC-*p*-NO_2-PheGly and ammoniated to BOC-*p*-NO_2-PheGly-amide, which was deblocked by being stirred with a mixture of dioxane and aqueous HCl. Reduction of *p*-NO_2-PheGly-amide was performed with diborane by using the procedure described above for preparation of *p*-NO_2-Bz-dien. NMR revealed this product to be identical with **10**.

Characterization. Intermediates and products of the ligand syntheses could best be identified by NMR and electron-impact mass spectroscopy. High-resolution proton resonance spectra at 500 MHz obtained over a range of solution pH values were required to provide primary evidence for structure. The most important NMR spectra are included as supplementary material.

The low volatility of zwitterionic nitriloacetates has heretofore rendered mass spectroscopy of limited use, but by converting all carboxylates to their trimethylsilyl esters, we were able to detect both molecular ions and interpretable fragmentation patterns. Mass spectral data are tabulated as supplementary material. Elemental analyses, chromatography, and optical spectroscopy further established the molecular structure. Thin-layer chromatography was principally useful for monitoring completeness of reactions, while semipreparative HPLC proved invaluable for purification of indium-111 radiolabeled antibody; chromatograms obtained by dual UV and radiometric detection provided estimates of specific activity, which were later confirmed by separate protein analysis and scintillation counting.

Mass Spectroscopy. It proved possible to obtain useful and interpretable electron-impact mass spectra for polycarboxylate ligands only by exhaustive trimethylsilylation of their carboxylates.

The M^+ peak for the tetrasilyl ester of *p*-NO_2-Bz-EDTA is clearly measured at 715 amu. The characteristic loss of one methyl generates the fragment at 700 amu. Loss of CO_2Me_3Si is observed at 598 amu, while cleavage of *p*-nitrobenzyl ($C_7H_6NO_2$) produces a fragment at 579 amu. A signal at 425 amu is due to loss of a silylated methyleneiminodiacetic acid fragment ($C_{11}H_{24}NO_4Si_2$), while the peak at 290 amu is from the residual fragment.

p-NH_2-Bz-EDTA exhibits a similar patern with loss of CH_3 seen at 670 amu, loss of *p*-aminobenzyl at 579 amu, and loss of silylated methyleneiminodiacetic acid at 396 amu. A complication in the mass spectrum is seen for *p*-NH_2-Bz-EDTA because two compounds are actually present after the silylation procedure. Silylation at the aniline occurred, in part, in addition to tetrasilylation of the four carboxylates. This gave rise to an M − 15 peak at 742 amu for loss of CH_3 as well as the peak at 468 amu from loss of the silylated methyleneiminodiacetic acid (M − 289).

p-NO_2-Bz-DTPA provided upon silylation a fragmentation pattern clearly predictable in light of data for the similarly substituted EDTA. A weak but discernable M^+ is recorded at 888 amu. The loss of CH_3 produces the fragment at 873 amu. Loss of CO_2Me_3Si is seen at 771 amu, with cleavage of *p*-nitrobenzyl ($C_7H_6NO_2$) generating a fragment at 752 amu. The characteristic loss of a silylated iminodiacetic acid fragment ($C_{11}H_{24}NO_4Si_2$) is seen at 598 amu. Loss of a silylated ethyleneiminodiacetic acid fragment ($C_{12}H_{26}NO_2Si_2$) is found as well as 584 amu. Splitting the molecule nearly in half produces fragments at 463 and 425 amu with the larger fragment being a silylated (*p*-nitrophenethyl)iminodiacetic acid ($C_{18}H_{39}N_2O_6Si_3$) with the remainder of the molecule ($C_{13}H_{29}N_2O_6Si_2$) providing the lower molecular weight fragment.

Linkage of Ligands to MoAb B72.3. Four chemically modified ligands were coupled to murine monoclonal antibody B72.3. A summary of reaction conditions, yield, and retention of immunoreactivity for the B72.3 conjugates is presented in Table I.

Labeling with the isothiocyanate ligands and **1** and **2** was effected by reaction with protein amine groups to form thiourea bonds. The coupled antibodies could be stored for months without loss of ^{14}C-tagged ligand as measured by dialysis against saline buffers in the pH range 5–8.

For each ligand studied, when about one chelate is linked per antibody molecule, no loss of immunoreactivity was detected, but when more than three ligands are coupled, loss of immunoreactivity as measured by competitive binding is seen for all conjugates (Table I). Reduction of immunological activity was most severe for MoAb coupled to CA-DTPA. Preparations of MA-DTPA that contain a statistical distribution of 0–2 DTTA chelates/molecule were less harsh. Use of an organic solvent to introduce CA-DTPA or MA-DTPA into coupling reactions always caused an instantaneous clouding of the solution, which cleared rapidly. Attempts to react solid CA-DTPA with B72.3 always resulted in loss of immunoreactivity.

Recoveries of coupled MoAb were good considering the small reaction volumes. Most of the loss was to the dialysis bag. If larger quantities were employed, 1–3 mL, usually 85–95% of the MoAb, was recovered.

^{111}In Labeling of the Antibody. Only chelate-linked preparations of B72.3 that retained 100% immunoreactivity were labeled. Commercial $^{111}InCl_3$ in dilute HCl was first be brought to 0.5 M [acid] to breakup hydrolysis products. Thereafter, sodium acetate solution was used to lower acidity to pH 4–5.5 followed by immediate addition of chelate-linked protein. When 5 mCi/0.1

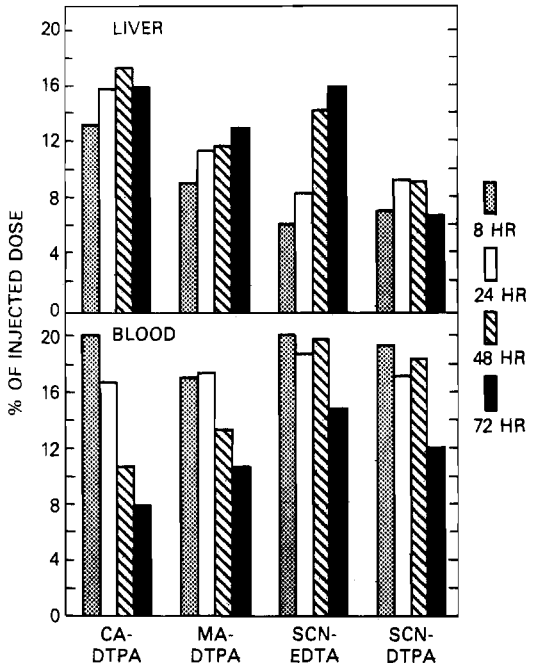

Figure 1. Changes in liver and blood uptake (measured as percent injected dose per gram) with time following injection into LS-174T-tumor-bearing athymic mice of B72.3 antibody labeled with ^{111}In by use of the chelates indicated. See Chart II and text for abbreviations.

mL of ^{111}In and 0.5 mg/0.2 mL antibody were reacted, specific activities of 5 mCi/mg were obtained. No loss of immunoreactivity could be detected as a result of the ^{111}In incorporation step.

Purification of ^{111}In-Labeled B72.3. Four purification protocols were tested to remove uncomplexed ^{111}In from labeling reactions. Initially, the reaction mixture after several hours incubation was separated on a Sephadex G-50 gel column. A reasonable separation of protein from free ^{111}In was observed. Yet the labeled antibody produced biodistributions consistent with substantial uptake (vide infra) of label by transferrin.[56] We therefore further purified another sample of labeled MoAb by passing the protein-containing fractions from the gel separation through a 30-cm TSK-3000 size exclusion column on an HPLC while monitoring both protein and ^{111}In by dual UV and radiometric detection. Excellent separation was seen between labeled IgG and all other radioactivity, including protein aggregates, which contained 10–20% of radioactivity. When the initial reaction solution was introduced directly into the HPLC without prior purification, good aggregate separation was again seen, but some tailing between protein and low molecular weight ^{111}In was detected.

We also tested a method that has been suggested[49] for in situ separation of all ^{111}In not complexed by chelates linked to antibody from the MoAb by in situ complexation with EDTA. Thereby, after the labeling reaction, a solution of 0.1 mM EDTA in citrate buffer was added to the reaction and incubated for 1 h prior to use in biological studies.

Biological Results. Biodistributions. Female athymic mice bearing human colon carcinoma were prepared for study by injection[40] of the LS-174T cell line, which expresses the high molecular weight TAG-72 antigen that binds B72.3. Fourteen days after inoculation, mice developed tumors 0.5–1.0 cm in diameter. Forty animal sets were injected with about 10 μCi of ^{111}In linked to B72.3 by each of the chelates of Table I and purified by the two-column procedure described above and were sacrificed at selected time intervals. Necropsy studies provided tissue distributions.

In Figure 1, the time course of the deposition of ^{111}In is recorded as the percent of injected dose per gram of tissue. It is particularly noticeable that uptake in the liver is initially low and remains so

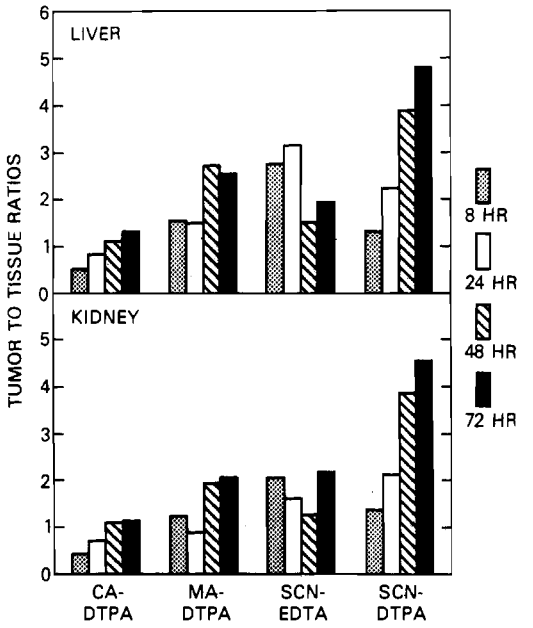

Figure 2. Changes in tumor to liver and blood ratios with time following injection of antibody B72.3 labeled with ^{111}In by use of the chelates listed.

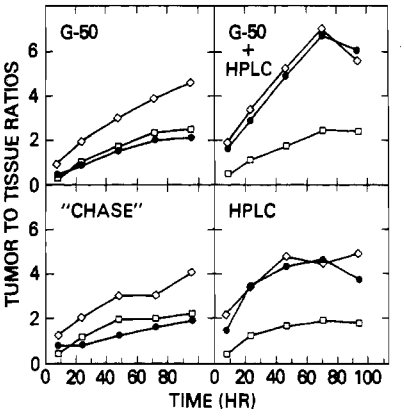

Figure 3. Time course of tumor to tissue ratios following injection of ^{111}In-SCN-Bz-DTPA-B72.3 purified by the four protocols indicated (see Experimental Section): (□) blood; (●) liver; (◇) spleen.

only for mice injected with ^{111}In-SCN-Bz-DTPA-B72.3. Also note the rapid blood clearance of MoAb labeled by using CA-DTPA.

Since nuclear imaging depends on tumor uptake relative to surrounding tissues, concentrations of label in five solid organs were obtained and ratios of percent injected dose per gram were formed for tumor relative to other organs. In Figure 2, we show the time course of tumor:liver (T:L) and tumor:kidney (T:K) ratios. When the *p*-SCN-Bz-DTPA chelate was used, T:L increased markedly over 72 h, while T:L dropped over the same time when the corresponding EDTA chelate was employed. After the first day, T:L did not change markedly for mice injected with ^{111}In-labeled B72.3 coupled to MA- and CA-DTPA. A similar pattern was seen for T:K as well as for those of other organs, particularly the spleen and blood.[51]

In experiments separate from the above, four purification protocols were tested for efficacy by measuring ratios of tissue distributions of radioisotopes as recorded in Figure 3. The "chase" method is seen to provide the least satisfying data, with very low T:L. Sephadex G-50 chromatography produced slightly better results, but clearly the highest T:L was obtained by HPLC separations. We had earlier[57] observed that columns containing

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Table I. Coupling of ^{14}C Chelates to MoAb B72.3

chelate	molar ratio of chelate:antibody in linkage reaction[a]	chelate:antibody in coupled B72.3[b]	immunoreactivity retained,[d] %	antibody recovery,[c] %
CA-DTPA	2:1	0.9	100	65
	4:1	2.0	85	68
	8:1	3.1	61	72
	16:1	6.5	28	67
MA-DTPA	100:1	0.8	100	74
	200:1	1.4	100	70
	300:1	2.3	100	63
	400:1	3.3	90	60
p-SCN-Bz-EDTA	0.5:1	0.3	100	56
	1:1	0.6	100	52
	2:1	1.3	100	57
	3:1	1.4	84	55
	6:1	2.8	36	59
	8:1	3.7	20	56
p-SCN-Bz-DTPA	1:1	0.4	100	72
	3:1	0.9	100	74
	6:1	2.0	95	75
	12:1	2.7	75	74

[a] Protein concentration for coupling reactions was 5 mg/mL with 0.2 mL used for *p*-SCN-Bz-EDTA and 0.5 mL for other chelates. [b] After chelation of ^{111}In, products are denoted, for example, as ^{111}In-*p*-SCN-Bz-DTPA-B72.3 for MoAb B72.3 coupled with *p*-SCN-Bz-DTPA and labeled by ^{111}In. [c] Based on measurement of absorbance at 280 nm, not corrected for absorbance of thiocyanate ligand aromatic group. [d] Immunoreactivity retained as measured by competitive binding assay.[51,70]

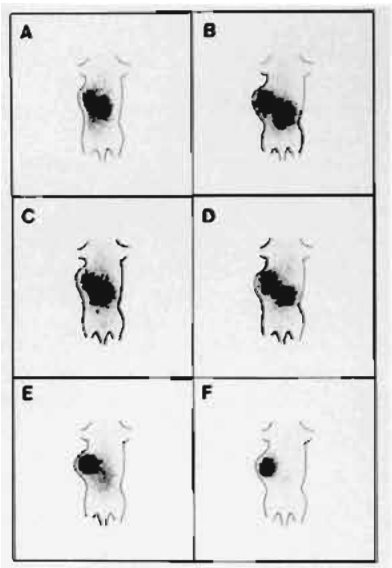

Figure 4. Scintigrams of nude mice (dorsal view) bearing LS-74T tumors obtained at 72 or 168 h after injection of B72.3 MoAb labeled with ^{111}In by use of four chelates: (A) In-CA-DTPA-B72.3, 72 h; (B) ^{111}In-*p*-SCN-Bz-EDTA, 72 h; (C) ^{111}In-MA-DTPA-B72.3, 72 h; (D) ^{111}In-MA-DTPA-B72.3, 168 h; (E) ^{111}In-*p*-SCN-Bz-DTPA-B72.3, 72 h; (F) ^{111}In-*p*-SCN-Bz-DTPA-B72.3, 168 h.

stacked ion-exchange resins and G-50 gel gave good separations, but in view of the superior HPLC results, those were not employed in the current study.

Immunoscintigraphy. Posterior whole body γ-camera images of athymic mice bearing LS-174T tumors or control TAG-72 antigen negative melanoma tumors[40,51] were obtained by using a pinhole collimator. Scintigraphs taken post-injection iv of 10–20 μCi ^{111}In linked to MoAb B72.3 by use of the four chelates of Table I are shown in Figure 4. Each antibody preparation was radiolabeled according to the ^{111}In protocol described above and purified both by column chromatography on Sephadex G-50 Gel and HPLC. Images were obtained daily for a period of 7 days. Tumors were visualized after 24 h, but for all chelates in the study, clearer images of tumors were obtained after 72 h. Specificity of the antibody was demonstrated by lack of specific uptake of ^{111}In in mice bearing antigen negative tumors[51] (images not shown). Numerous controls, including demonstration that an irrelevant MoAb of the same class as B72.3 does not localize in tumor were previously reported.[40]

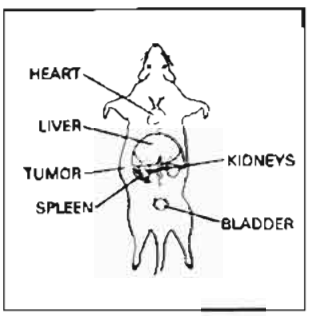

Figure 5. Anatomy of a tumor-bearing mouse, dorsal view.

The scintigraphs of Figure 4 provide a direct comparison of utility for imaging of the several ligands examined. The low T:L seen for CA-DTPA is reflected in Figure 4A. By reference to the anatomy of the mouse, Figure 5, we observe that liver and spleen images are more intense even than that of the tumor. In Figure 4B,C, we see substantial liver uptake in the 3-day images obtained by using the EDTA and MA-DTPA ligands, whereas an image obtained with *p*-SCN-Bz-DTPA (Figure 5E) clearly shows less abdominal uptake. The continuous increase in tumor:organ ratios observed over 7 days for the intact DTPA chelate further results in the remarkably sharp tumor visualization of Figure 4F as compared to that of Figure 4D obtained from MoAb linked to MA-DTPA.

Discussion

In the course of developing protocols for ^{111}In labeling of MoAb B72.3 for imaging of human colorectal carcinoma, we found it necessary to perform a careful, systematic investigation of the relative utility of DTTA and EDTA chelates and of the methodologies used for coupling to MoAb. Our dissatisfaction with images obtained for all available ligands prompted us to devise a synthesis of *p*-SCN-Bz-DTPA for linking B72.3 to an intact DTPA. Inasmuch as we had developed a preparation for *p*-NH_2-Bz-EDTA, we modified and extended that pathway for this study (Chart II) to obtain *p*-SCN-Bz-EDTA and *p*-SCN-Bz-DTPA.

Our rationale for expecting that intact DTPA chelates would be superior for use in vivo was based on several observations. First, kinetic studies indicated that loss of indium from EDTA itself was catalyzed by amino acids in solution.[34,35] We reasoned that the extra carboxylate(s) available with DTPA, vs. DTTA or EDTA would serve as intramolecular competition for metal binding sites of dissociation catalysts since the coordination number

of indium is unlikely to be more than seven. Secondly, we expected that indium–DTPA was simply more thermodynamically stable than the corresponding DTTA complex,[60] and we know they are more stable than those of EDTA.[61] Additionally, we noted the report which stated that a protein-linked DTTA was less inert to loss of radioindium than DTPA.[30] It was our further hypothesis that some of the current problems being encountered in clinical trials[9-11] and animal model studies[49,62-65] with ^{111}In-labeled MoAb might be due to insufficient chemical stability of the complex and/or inadequate purification of radioimmunological products.

Methods for covalently linking chelates to antibody were first reviewed. Since both isothiocyanate and anhydride chelates could be linked to B72.3 without loss of immunoreactivity, it is evident that these chemical modes of linkage are mild enough to be useful. We note that attachment of two or more CA-DTPA ligands caused much greater loss of activity in comparison to MA-DTPA (Table I). This could be from internal or external cross-linkage of protein possible to a great extent only for the dicyclic dianhdyride. Use of organic solvent for introduction of anhydrides into antibody solution always caused instantaneous clouding of solutions, which clears rapidly. We speculate that precipitation might be deleterious to the MoAb in some subtle way not measured by RIA. Our attempts to react weighed, solid CA-DTPA with B72.3 always resulted in loss of immunoreactivity, but other workers[66] have reported success by using solid films. Hydrolysis of the dianhydride releases three proton equivalents, which could create conditions of high local acidity near the bulk solid, which could damage the MoAb. In our hands, the preparation of films led to inconsistent results in the number of chelates linked to an antibody because of partial hydrolysis of CA-DTPA.[54] No attempts to form chelate conjugates by use of diazonium salts or by carbodiimide coupling to carbohydrates have resulted in useful products, so our current and future efforts will employ isothiocyanates.

Procedures for inserting ^{111}In into chelates linked to proteins have been examined, but little careful attention has heretofore been given to methods for purification of non-chelate-linked ^{111}In from labeled protein, although the need had been noted.[57] The "free" ^{111}In may be present in several forms and places. First, ^{111}In might be ionic or hydrolyzed, present in non-antibody-attached chelates in solution, or it might be weakly and adventitiously linked to protein at natural metal binding sites on the molecular backbone. Whatever its source, ^{111}In in vivo but not in very strong chelates will be rapidly bound by transferrin and will therefore result in undesirable localization in normal tissues as detected in images.[56] The results in Figure 3 show that, by the methods of this study, simple addition of excess EDTA was not sufficient to remove all unreacted ^{111}In, which deposited in the liver and led to low T:L ratios. The rationale[49] for this "chase" method was that EDTA would scavenge all ^{111}In not tightly complexed and be eliminated rapidly from the body through the kidney.[67] The time course data of Figure 3 would seem to invalidate that hypothesis. Commonly employed gel chromatography columns produced a modestly improved product, but clearly the best separations and corresponding T:L tissue distributions were obtained by using size-exclusion HPLC. It is important to note that only HPLC removes high molecular weight aggregates, which invariably seem to form in ^{111}In-labeling reactions.

Dual purification by both G-50 column and HPLC was employed to do a direct comparison of tissue distributions for B72.3 conjugated to the several chelates chosen for study (see Table I) and labeled with ^{111}In. Evaluation of tissue uptake data for ^{111}In-SCN-Bz-DTPA-B72.3 and ^{111}In-SCN-Bz-EDTA-B72.3 (Figure 1) shows that the former ligand better retains the isotope with the antibody. The DTPA conjugate is found in the liver in continuously low amounts while, for EDTA-linked B72.3, the amount in the liver increases monotonically over 72 h. Leakage from ^{111}In-MoAb formed with use of EDTA or DTTA ligands is about 10% per day,[68] which explains the increase in dose outside the tumor. Similar uptake was seen for other solid organs.[51] Since liver is heavily perfused with blood, the amount there for even the best chelate may simply reflect the percentage of the dose due to circulating MoAb. In Figure 1, we also note that the unusually rapid rate of clearance of CA-DTPA-linked B72.3 from the blood is evidence of damaged MoAb, perhaps from internal cross-linking or loss of label from the triacetic acid chelate formed when both anhydrides are amine linked.

Further evidence of the need to use the strongest chelate, the mildest coupling, and best available purification technique is shown by the T:L and T:K data of Figure 2. As antibody localizes in tumor over time, one might expect that the ratios would increase. However, continuous leakage of ^{111}In from chelate to blood, followed by transport by transferrin to liver and clearance in the kidney, occurs for all but B72.3 labeled with the intact DTPA and accounts for the poor T:L and T:K for the other chelates.

Most importantly, images taken 7 days after injection (Figure 4) were markedly superior for MoAb labeled by using *p*-SCN-Bz-DTPA. The clear implication is that this chelate better retained ^{111}In in vivo. Again, upon clearance, improved images were seen. While these results can be considered to be of general importance for solid tumor imaging with MoAb, highly vascularized leukemic or lymphatic tumors exhibit more rapid pharmacokinetics of antibody binding and metabolism rendering relative stability of the chelates of less import for ^{111}In scintigraphy.[2,6,49]

Available data do not allow us to precisely determine the chemical factors that result in the improved retention of ^{111}In by intact DTPA.[60] Certainly, increased thermodynamic stability is important. Further kinetics and thermodynamics research is clearly in order, especially since our results are not those expected from "serum stability" studies.[69] Therein, rates at which several EDTA and DTPA chelates lost ^{111}In to transferrin in human serum were measured. It was reported that 1-substituted EDTA ligands, such as *p*-SCN-Bz-EDTA, were the most inert, while DTPA and a protein labeled by use of MA-DTPA lost ^{111}In more rapidly. By implication, this order of reactivity was thought to be maintained in vivo, but we found that ^{111}In linked to B72.3 by *p*-SCN-Bz-DTPA was lost slowest. We attempted to reproduce the serum kinetics studies[69] but found that substantial ^{111}In precipitated and was not incorporated into transferrin or other serum proteins. We note that our in vivo data make it apparent that inertness in serum is a necessary but not a sufficient requirement for inertness in vivo and an uncertain predictor of relative rates of dissociation.

Current results indicate that radiometal-labeled MoAb could become a useful clinical tool for diagnosis and treatment of cancer. When care was taken with the chemistry, tumor uptake was achieved rapidly and persisted, whereas nontumor accumulations were lower and cleared more rapidly. Since cytotoxic α- or β-particle-emitting radionuclides may also be chelated by DTPA, radioimmunotherapy might also be possible. Our current studies of selective in vitro cell killing by employing the 1-h half-life α-emitter ^{212}Bi coupled to an antibody to adult T-cell leukemias have proven successful.[70] α-Particle-emitting radionuclides appear to be ideal for therapy since their very powerful radiation is delivered over a very short range, about 10 cell diameters for ^{212}Bi,

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thus minimizing the dose to surrounding normal tissues. We note that *p*-SCN-Bz-DTPA is the most stable chelate available for ^{212}Bi. Finally, current studies[71] with the β-emitter ^{90}Y indicate a pattern of stability for the various chelates similar to that reported here for ^{111}In. A detailed investigation of in vivo stability of protein-linked therapeutic radionuclides is under way.

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Supplementary Material Available: Tables of analytical and mass spectral data and a figure showing NMR spectra (3 pages). Ordering information is given on any current masthead page.

(71) Strand, M.; et al., private communication.

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X-ray Absorption Studies of the Purple Acid Phosphatase from Beef Spleen

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Iron K-edge near-edge (XANES) and extended X-ray absorption fine structure (EXAFS) spectra have been measured for the purple acid phosphatase from beef spleen and for several oxo-bridged model complexes. The XANES show a shift of the absorption edge to lower energy by 2.0 eV upon reduction of the purple form of the enzyme to the pink form, consistent with reduction of one of the iron atoms. Fourier transforms of the EXAFS data of the purple form of the enzyme show three peaks, assigned to Fe–O(N) (first shell), Fe--Fe and Fe--P or Fe--C (second shell), and Fe--C(N) (imidazole, third shell) scatterers, in order of increasing distance. Best fits of the individually back-transformed peaks using theoretical functions are consistent with the indicated assignments. The Fe--Fe distance of 3.00 Å is consistent with a bridged binuclear iron center in the enzyme. Due to the presence of short Fe–O(tyrosine) linkages at 1.8–1.9 Å, direct evidence for a bridging oxo group at ca. 1.8 Å could not be obtained. The observed Fe--P distance of 3.06 Å is most consistent with the presence of a phosphate as a monodentate ligand to one iron atom in the purple (oxidized) form of the enzyme.

Purple acid phosphatases are characterized by their intense ($\epsilon \sim 4000$ M^{-1} cm^{-1}) absorption band at 510–550 nm.[5] Their presence in mammalian, plant, and microbial sources[5,6a] suggests that these enzymes are of primary importance in the regulation of the physiological level of inorganic phosphate and phosphorylated metabolites. The enzymes from beef spleen[6] and porcine uterine fluid[6d,7] both contain diiron centers, whereas the enzyme from sweet potato is reported to contain manganese.[8] Electron paramagnetic resonance spectra[6e,9] and magnetic susceptibility studies[9] of the enzyme isolated from beef spleen are consistent with the oxidized (purple) enzyme containing an antiferromagnetically coupled $[Fe^{III}]_2$ unit and the enzymatically active, reduced (pink) enzyme containing a spin-coupled mixed-valence Fe(III)–Fe(II) unit. A similar picture is now accepted for the porcine uterine enzyme.[10] The EPR spectra and magnetic properties of the semimet form of hemerythrin[11] are very similar to those observed in the purple acid phosphatase from beef spleen and porcine uterine fluid, suggesting the existence of a related binuclear unit in all three proteins. Results from resonance Raman studies have established the presence of tyrosine ligands to iron in both the porcine[12] and beef spleen[13] enzymes. On the basis of proton NMR spectra, both histidine and tyrosine groups are suggested to be bound to the iron of the porcine enzyme.[14] EPR and UV–visible absorption studies of phosphate binding indicate that phosphate binds near, if not at, the iron site.[7,9,15] Magnetic

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