hydrol were dissolved in 100–200 ml of glacial acetic acid. The solution was heated at refluxing temperature for 8 hr. A sample of the solution was added to cold water. The unreacted diphenylmethyl acetate present in the precipitate from incomplete reactions gave oils that would not solidify. Completed reactions yielded solid products or oils that eventually crystallized. These products also gave mainly one spot when developed on thin-layer plates. The cooled reaction mixture was added to cold water and the resulting solid was collected and washed with cold water. The products were dissolved in hot ethyl acetate and *n*-hexane was added until incipient turbidity. The compounds usually crystallized but a few intensely colored compounds did not. The latter required clarification with carbon, time, and concentration changes to initiate crystallization. All yields are reported for analytically pure compounds.

Compound **50** [Table V, 3-(diphenylmethyl)-1-indolepropionic acid] failed to crystallize and was chromatographed on a silica column with 1% acetic acid in ethyl acetate.

Structures of the alkylation products were established by elemental analysis and pmr spectra. The purity of the compounds was checked with thin-layer chromatography but the melting points were not calibrated.

5-Hydroxy-3-(diphenylmethyl)-2-indolecarboxylic Acid (43, Table IV). 5-Benzyloxy-3-(diphenylmethyl)-2-indolecarboxylic acid (8.7 g or 0.023 mol) in 500 ml of alcohol was heated at 40–50° with hydrogen at 50 psi over 5% Pd/C catalyst in a Parr high-pressure autoclave until there was no further drop in hydrogen pressure (3 hr). The solution was filtered and evaporated; the resulting product was crystallized from a mixture of ethyl acetate and *n*-hexane.

Supplementary Material Available. Elemental analysis for compounds **40–75** and Tables II, X, and XI will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number JMED-74-1298.

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Selective Binding of Metal Ions to Macromolecules Using Bifunctional Analogs of EDTA

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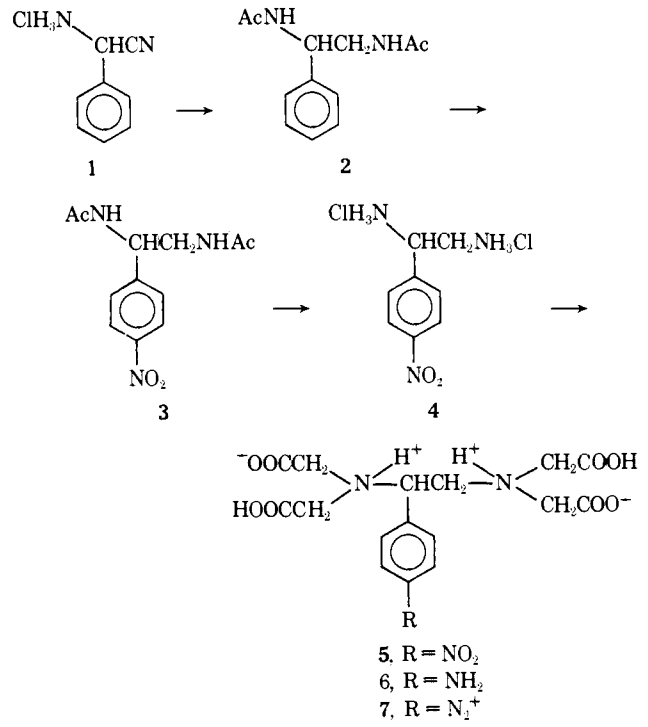
The synthesis of 1-(*p*-benzenediazonium)ethylenediaminetetraacetic acid, the coupling of this compound to proteins, and the binding of radioactive metal ions to the protein-bound chelating groups are described. This procedure provides a novel approach to the preparation of radiopharmaceuticals, permitting the separation of synthetic organic chemistry from radiochemistry. Azoproteins labeled with indium-111 are relatively stable *in vivo* and potentially useful for the detection and localization of tumors. Other chelating agents derived from 1-(*p*-aminophenyl)ethylenediaminetetraacetic acid may permit new applications of a variety of metal ions with useful physical properties to studies of biological systems.

Polyaminocarboxylate chelating agents such as ethylenediaminetetraacetic acid form stable complexes with the ions of many heavy metals. Since these metal ions possess a variety of useful spectroscopic and radioactive properties, the preparation of chelating agents whose complexes interact, in some selected manner, with biological macromolecules may lead to new applications of metal ions as probes of biological systems.

As an intermediate in the preparation of molecules with the dual properties mentioned above, 1-(*p*-aminophenyl)ethylenediaminetetraacetic acid (**6**, Scheme I) has several advantages. In principle, the aromatic amino group of this compound can be acylated, alkylated, or otherwise modified to form either biologically active derivatives or covalent labeling reagents. The specific interaction between 1-(*p*-nitrophenyl)ethylenediaminetetraacetic acid

(5) and an immunoglobin G molecule has been described previously.[1] The labeling reagent 1-(*p*-benzenediazonium)ethylenediaminetetraacetic acid (7) may be prepared by diazotization of 6.†

Scheme I



Results

As illustrated in Scheme I, 1-phenylglycinonitrile hydrochloride (1) was converted to *N,N'*-diacetyl-1-phenylethylenediamine (2) by catalytic hydrogenation in acetic anhydride.[3] The aromatic ring of 2 was nitrated at the para position with 90% HNO_3 at −40°, and the product (3) was refluxed in mixed acetic acid-hydrochloric acid to yield diamine 4. Compound 4 was allowed to react with 4 equiv of iodoacetate to give aminopolycarboxylic acid 5, and the aromatic nitro group of 5 then was reduced (H_2, Pd/C) to yield 6. Aromatic amine 6 was diazotized to form 7, which then was coupled to the proteins serum albumin (human) and fibrinogen (bovine). The addition of $^{111}InCl_3$ to 1% solutions of each azoprotein (in 0.1 *M* sodium citrate, pH 6) led to rapid attachment of the indium ions to the protein-bound chelating groups. The metal binding was monitored by the perturbed angular correlations technique and substantiated by *in vivo* distribution studies in rabbits and in mice.

γ-Ray Perturbed Angular Correlations.‡ The radioactive decay scheme of ^{111}In includes the sequential emission of two γ-rays from each nucleus. There is an angular correlation between the directions of propagation of these two γ-rays. Interactions between the radioactive nucleus and its surroundings can perturb this angular correlation in well-characterized ways. For ^{111}In, the perturbed angular correlation depends on the rotational correlation time, τ_c, and the magnitude, e^2qQ, of fluctuating electric field gradients at the nucleus.[5] When a free indium ion binds to the chelating group of an azoprotein, both τ_c and e^2qQ can change, resulting in a decrease in the measured value of the integral perturbation factor, $\overline{G_{22}(\infty)}$.

†For a preliminary report, see ref 2a. The synthesis was abstracted, in part, from Sundberg's thesis; see ref 2b.

‡For a recent review, see ref 4.

Table I. *In Vitro* Studies of Indium Binding by Perturbed Angular Correlations

Sample[a]	$\overline{G_{22}(\infty)}^b \pm$ S.D.
$^{111}InCl_3$[c]	0.60 ± 0.01
$^{111}InCl_3$[c] + compd 5[d]	0.58 ± 0.02
$^{111}InCl_3$[c] + fibrinogen[e]	0.57 ± 0.02
$^{111}InCl_3$[c] + albumin[e]	0.59 ± 0.02
$^{111}InCl_3$[c] + azofibrinogen[e,f]	0.37 ± 0.02
$^{111}InCl_3$[c] + azoalbumin[e,g]	0.40 ± 0.02

[a] All samples in 0.1 *M* sodium citrate, pH 6, room temperature. [b] Integral perturbation factor. See text. [c] Carrier-free, 10 μCi. [d] 10^{-4} *M*. [e] 10 mg/ml. [f] Reacted with a two- to threefold excess of 7. [g] Reacted with an equimolar amount of 7.

The integral perturbation factor was measured for solutions containing carrier-free $^{111}InCl_3$ and native proteins, azoproteins, or chelating agent 5. Results are given in Table I. Under the conditions of these experiments, the indium complexes of low molecular weight yield $\overline{G_{22}(\infty)} \simeq 0.6$, suggesting that the indium citrate complex and the indium chelate with 5 have comparable correlation times and quadrupolar interactions. The values of $\overline{G_{22}(\infty)}$ obtained with solutions of the *native* proteins are quite similar to those of the low-molecular-weight complexes; however, the values for the azoprotein solutions are distinctly different, indicating that the indium ions are bound to the chelating groups of the azoproteins. This binding is complete within a few minutes after the reactants are mixed. Binding has been confirmed by dialysis of indium-labeled azoalbumin.

Metal-Exchange Studies in Rabbits. For 6 days following injection of ^{111}In-labeled azoalbumin into adult New Zealand rabbits, the distribution of radioactivity among the plasma proteins was investigated by electrophoresis of plasma samples. As shown in Figure 1, the injection of $^{111}InCl_3$ leads to rapid and nearly quantitative binding of ^{111}In by transferrin, but 6 days after the injection of ^{111}In-labeled azoalbumin, the major portion of the radioactivity remains in the albumin fraction.

Tumor-Bearing Mice. BALB/c mice with a "KHJJ" tumor implanted in the flank were injected with ^{111}In-labeled azoalbumin, ^{111}In-labeled azofibrinogen, or ^{131}I-labeled albumin, and the organ distribution and tumor uptake of radioactivity were investigated. Results, determined 24 hr after injection, are given in Table II. For the ^{111}In-labeled azoproteins, both the tumor uptake and the ratio of the concentration of radioactivity in the tumor to the concentration of radioactivity in the organs compare favorably with a number of other agents studied in the same model system.[6]

Discussion

The mild reaction conditions and rapid metal binding described here suggest that this approach will provide a useful means for labeling biologically active molecules with short-lived radionuclides. The use of plasma proteins labeled with radioiodine for the detection and localization of tumors is well established.[7] Compared to iodination, the approach described here possesses substantial advantages in the potential variety of accessible radionuclides and reactive groups and in the separation of synthetic organic chemistry from radiochemistry. The tumor uptake and organ distribution observed in tumor-bearing mice would be favorable for scanning studies on human patients, and the radioactive properties of indium-111 are

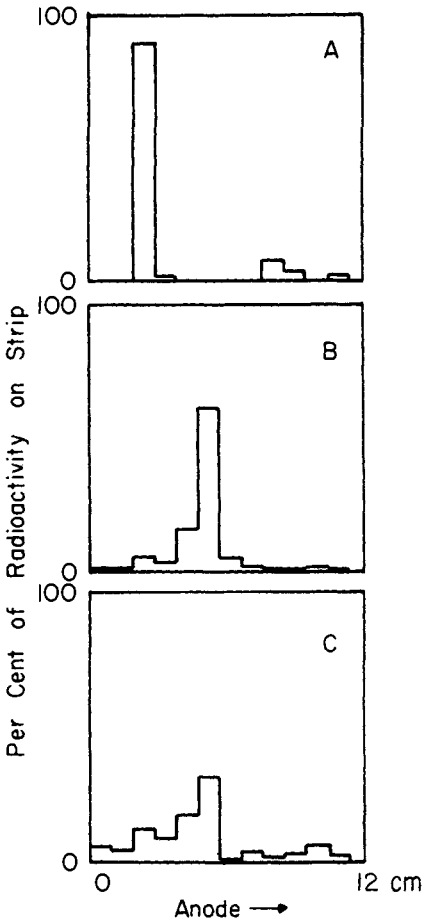


Figure 1. Cellulose acetate electrophoresis of rabbit plasma: (A) 1 day after injection of $^{111}InCl_3$ (radioactivity appears almost quantitatively in transferrin band); (B) 1 hour after injection of ^{111}In-labeled azoalbumin; (C) 6 days after injection of ^{111}In-labeled azoalbumin (6% of the radioactivity migrated toward the cathode).

ideal for providing scans up to 1 week after administration, with a minimum of radiation exposure to the patient.[8]

Because of the kinetic inertness or large conditional stability constants of their EDTA chelates, the ions of Co, Ni, Sn, Cr, Fe, Zr, Hf, Y, Al, In, Ga, Bi, Hg, Rh, Pd, Ir, Os, Ru, Th, U, the lanthanides, and the actinides may be useful in future applications of the chelating agents described here. These metal ions exhibit useful spectroscopic and radioactive properties, such as observable epr spectra, the production of line-broadening and chemical shifts in nmr spectra, electronic absorption, scattering of electrons and X-rays, the emission of correlated γ-ray cascades, and a variety of radioactive lifetimes and nuclear radiations.

Experimental Section

A. Chemical Procedures. Reagents. Citrated bovine fibrinogen (fraction I) was obtained from Sigma, St. Louis, Mo. It was purified by the procedure of Blombäck and Blombäck[9] to fraction I-4 which, by spectrophotometric assay, was 98% clottable with thrombin. Human serum albumin (crystallized and lyophilized) was also from Sigma. Human serum albumin labeled with iodine-131 (Albumotope) was obtained from E. R. Squibb and Sons, New Brunswick, N.J., as a 1% solution in isotonic phosphate buffer, pH 7.5 (0.49 mCi/ml).

Carrier-free $^{111}InCl_3$ (in 0.05 *N* HCl, 0.9% NaCl) was obtained from Medi+Physics, Emeryville, Calif. It was purified by eluting with 0.2 *N* HCl from a 0.7 × 15 cm column of Bio-Rad AG 1-X8 anion-exchange resin and evaporating the fractions in nalgene beakers.

Table II. Distribution and Uptake of Labeled Macromolecules in BALB/c Mice with KHJJ Tumor

Organ	% of injected radioactivity per gram[a]		
	^{111}In–azoalbumin	^{111}In–azofibrinogen	^{131}I–albumin
Blood	4.6 ± 0.6	6.7 ± 1.1	6.8 ± 1.6
Lungs	3.4 ± 0.6	3.1 ± 0.3	3.4 ± 1.2
Liver	8.6 ± 0.3	9.2 ± 0.5	1.1 ± 0.3
Spleen	3.3 ± 0.1	7.0 ± 0.7	0.7 ± 0.2
Kidneys	11.4 ± 0.1	18.1 ± 1.9	2.4 ± 0.6
Tumor	9.7 ± 0.3	8.1 ± 0.8	3.3 ± 1.0
Muscle	0.8 ± 0.2	0.8 ± 0.2	0.9 ± 0.2
Bone	2.0 ± 0.4	1.9 ± 0.4	1.0 ± 0.5
Brain	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
Skin	2.4 ± 0.4	2.0 ± 0.3	*b*

[a] Mean ± S.D. [b] Not determined.

All syntheses employed purest commercially available reagents. Melting points are uncorrected. Elemental analyses were performed by the Stanford University Microanalytical Laboratory and in all cases were within ±0.4% of the theoretical values.

1-Phenylglycinonitrile Hydrochloride (1). This was prepared according to Steiger[10] after commercial products proved unsatisfactory. The hydrochloride was precipitated from benzene solution with gaseous HCl. The yield was 30–40%, mp 171–172°.

***N,N'*-Diacetyl-1-phenylethylenediamine (2).** After treatment with acetic anhydride,[3] 2 g of No. 28 Raney active nickel catalyst (W. R. Grace & Co.) was added to 33 g (0.20 mol) of 1, 25 g (0.30 mol) of sodium acetate, and 250 ml (2.65 mol) of acetic anhydride in a 500-ml Parr bottle. This was placed in a Parr hydrogenation apparatus and agitated at 50° under hydrogen at 45 lb/in.^{2} for 3 hr. Fresh catalyst (1 g) was added, and the reaction was continued for 3 hr, when hydrogen uptake ceased. The mixture was filtered, and the solvent was removed under reduced pressure. The product was extracted from the residue with several portions of boiling ethyl acetate. On reducing the volume and cooling the ethyl acetate solution, 28.8 g (67%) of **2** was obtained (mp 155–156°).

***N,N'*-Diacetyl-1-(*p*-nitrophenyl)ethylenediamine (3).** Compound **2** (3 g, 0.014 mol) was added slowly to 10 ml of 90% HNO_3 at −40°. After stirring 5 hr at −40°, the solution was poured over ice and neutralized with $NaHCO_3$. The product was extracted into ethyl acetate, and this was dried with $MgSO_4$ and then evaporated to dryness under reduced pressure. The product was recrystallized from acetone–hexane, yielding 2.2 g (61%) of **3**, mp 178–180°. *Anal.* ($C_{12}H_{15}N_3O_4$) C, H, N.

1-(*p*-Nitrophenyl)ethylenediamine Dihydrochloride (4). A solution of 4 g (0.015 mol) of **3** in a mixture of 20 ml of glacial acetic acid and 30 ml of concentrated HCl was heated at reflux for 24 hr and then cooled in ice.[3] Upon filtration, 2.5 g (66%) of the crystalline hydrochloride **4** was collected. *Anal.* ($C_8H_{13}N_3O_2Cl_2$) C, H, N.

1-(*p*-Nitrophenyl)ethylenediaminetetraacetic Acid (5). A solution of 0.61 g (2.4 mmol) of **4** and 2.1 g (11.3 mmol) of iodoacetic acid in 10 ml of H_2O was held at 45° for 8 hr, with the pH maintained at 10–11 by addition of 7 *M* KOH. The solution then was acidified to pH 1 with concentrated HCl and kept at 4° for 4 days. The crude crystalline product was collected and dissolved in a minimum volume of aqueous NaOH. This was applied to a 3 × 30 cm column of Bio-Rad AG 1-X8 anion-exchange resin in the formate form and eluted with a linear (0→5 *M*) gradient of formic acid.[11] The absorbance of the effluent was monitored at 280 nm; elution of **5** required 2–3 l. of eluent. The pure tetraacid crystallized in the fraction-collector tubes. In alkaline D_2O, the aromatic region of the 60-MHz nmr spectrum of **5** consisted of two doublets, 7.3 and 8.2 ppm downfield from Me_4Si: yield 300 mg (30%); mp 171–174°. *Anal.* ($C_{16}H_{19}N_3O_{10}\cdot H_2O$) C, H, N.

1-(*p*-Aminophenyl)ethylenediaminetetraacetic Acid (6). Nitro compound **5** (43 mg, 0.10 mmol) was dissolved in 50 ml of aqueous NaOH (such that the final pH was 9), and 29 mg of 10% Pd-on-charcoal catalyst was added. The mixture was stirred gently in an ice bath for 5 hr under 1 atm of hydrogen. The

catalyst was removed by filtration, and the filtrate was evaporated to dryness. In D_2O, the aromatic region of the 60-MHz nmr spectrum of **6** consisted of an aa'bb' pattern centered 7.0 ppm downfield from Me_4Si. The differences in the nmr spectra of **5** and **6** make it convenient to monitor the progress of the reduction by nmr. By this criterion, the reduction was quantitative. Amine **6** was stored in the dark at −15°.

Diazotization of 6. Preparation of 1-(*p*-Benzenediazonium)ethylenediaminetetraacetic Acid (7). The amine **6** obtained in the previous reaction (*ca.* 0.1 mmol) was dissolved in 0.5 ml of H_2O; 0.5 ml of concentrated HCl was added and the mixture was cooled to 0° in an ice bath. Cold 0.5 *M* $NaNO_2$ (0.25 ml, 0.125 mmol) was added dropwise with stirring, and the reaction mixture was stirred 1 hr at 0°. Urea (3 mg, 0.05 mmol) was added to destroy excess $NaNO_2$, and the reaction mixture was diluted to 10 ml with cold H_2O to form a stock solution, which was stable for months at −80°. This stock solution was standardized by coupling to resorcinol.[12]

Azoproteins. Conjugation of **7** with human serum albumin or bovine fibrinogen was accomplished by stirring overnight at 4° with a 2% protein solution in 0.01 *M* EDTA–0.12 *M* $NaHCO_3$, pH 8.1. Appropriate amounts of **7** stock solution were neutralized with solid $NaHCO_3$ before addition to the protein solutions. Albumin was allowed to react with an equimolar amount of **7**, while fibrinogen was allowed to react with a two- to threefold excess of **7**. The buffer ions and unbound reagent then were removed by extensive dialysis against 0.1 *M* sodium citrate buffer at pH 6 (prepared free of heavy-metal ions by dithizone extraction[13]).

Addition of Indium Ions to Azoproteins. Purified $^{111}InCl_3$ was dissolved in a minimum of 0.1 *M* citrate buffer (pH 6) and added to a solution of azoprotein in the same buffer. The binding of indium ions to the azoprotein was monitored by perturbed angular correlation measurements, as described in the text.

B. Perturbed Angular Correlation Measurements. Measurements were made using a four-detector γ-ray coincidence spectrometer with NaI(Tl) crystal scintillation detectors.§ The instrument incorporates eight scalers, permitting simultaneous determination of true and random coincidences in each of four quadrants. The coincidence resolving time was 600 nsec.

C. Biological Procedures. Distribution in Tumor-Bearing Mice. Following the injection of ^{111}In-labeled azoproteins into the tail veins of specially prepared BALB/c mice, the organ distribution and tumor uptake of radioactivity were determined. A tumor line, "KHJJ," derived from a primary mammary carcinoma arising in a mouse and maintained for over 100 transplant generations was used for the assay.[15] Transplantation was by subcutaneous implantation of tumor fragments about 1 mm in diameter into the flank. The studies were carried out after 14 days of growth, when the tumor had reached a size of about 1 cm^3. On histological examination, the tumor has a "carcinoma-like" pattern with a predominance of islands of round or polygonal malignant cells with little stroma and a generally undifferentiated appearance. After transplantation, the tumor takes in almost all animals and grows without metastasizing or killing the mice within 14 days. For the distribution assay, a volume of 0.2 ml containing approximately 0.1 μCi of labeled azoprotein was injected into the tail vein, using three mice per compound. After 24 hr, each mouse was anesthetized with ether, and blood was collected from the jugular vein into two preweighed capillary tubes. The mouse then was killed instantly by cervical dislocation and the major organs were excised. Samples of muscle, skin, bone (left femur plus marrow), tail, and tumor also were taken. All tissue samples were weighed immediately after excision and counted in a well-type scintillation counter. Results are given in Table II.

In Vivo **Metal-Exchange Studies in Rabbits.** For 6 days following the intravenous injection of ^{111}In-labeled azoalbumin into two adult New Zealand rabbits, the distribution of radioactivity among the plasma proteins was determined. Blood samples were taken from a marginal ear vein, and 5-μl samples of the plasma were electrophoresed on cellulose acetate strips (90 min, 18 V/cm) in 0.06 *M* veronal buffer, pH 8.6. Upon completion of the electrophoresis, the strips were cut into segments, which were counted in a well-type scintillation counter. Each strip was divided into a 5-cm "pre-origin" segment (extending from the cathode to the origin) and 12 1-cm segments extending from the origin to the anode. Results are given in Figure 1.

§For the description of a similar instrument, see ref 14.

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