Comparative Serum Stability of Radiochelates for Antibody Radiopharmaceuticals

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Serum incubation of monoclonal antibodies chelate labeled by DTPA, benzyl-EDTA and benzyl-TETA with ^{111}In, ^{57}Co, and ^{67}Cu demonstrated marked differences in their stability. In serum, ^{111}In-benzyl-EDTA-antibody was more stable than ^{111}In-DTPA-antibody. Cobalt-57 or ^{67}Cu chelated antibody were less stable than either ^{111}In chelated antibody; ^{67}Cu was only firmly attached to the antibody as ^{67}Cu-benzyl-TETA-antibody. The relative stability of the radiometal chelated antibodies was paralleled by the relative stability in serum of the radiometal chelates themselves. These in vitro studies suggest that in vivo behavior of metal chelates exposed to a complex protein environment cannot be predicted by classical equilibrium constants.

As a result of hybridoma technology (*1*), monoclonal antibodies offer an ever increasing array of molecules designed to react specifically against tumor cells, bacterial cells, myosin, and other biologic entities. Many investigators have realized the potential of these molecules as substrates for the development of radiopharmaceuticals for specific diagnostic imaging and selective delivery of radiation for cancer therapy (*2–5*). Various methods for radioiodination and indium-111 (^{111}In) chelate-labeling have been applied to monoclonal antibodies. Several products of these labeling methods have been evaluated in mice and a few have had evaluations in patients with cancer. Although the results have been promising (*4,6,7*), the same studies have also provided evidence that the radiolabel and the radiolabeling method substantially affects the radiopharmaceutical stability, kinetics, and biodistribution.

A number of attractive radionuclides for radioimmunoimaging or radioimmunotherapy fall in the general classification of radiometals, such as ^{111}In, technetium-99m, copper-67 (^{67}Cu), mercury-197, yttrium-90, and palladium-109 (*8–11*). To effectively utilize these radionuclides it is necessary to assure the stability of

the radiochelate in the protein environment with which it will interact in blood and extravascular tissue spaces. If the radiolabel is prematurely lost as free metal because of competition from serum components, or if the radiochelate is removed prematurely from the antibody and excreted through the kidneys, both imaging effectiveness and radiotherapeutic potential are diminished. The body's radiation burden may be increased or diminished depending on whether the radiometal is lost in a form which is quickly removed by the kidneys, or merely transferred to serum proteins which may make it available to other metabolic sites. Evaluation of the stability of a radiolabeled antibody in vivo is complicated by the variety of metabolic pathways available to the antibody and its radiolabel. We have, therefore, studied the stability of radiometal chelates and radiometal chelate-labeled antibodies in vitro in a serum environment.

The challenge of attaching radiometals to proteins has been approached by the use of various bifunctional chelates (*12–18*). Popular agents to date for this purpose include the mixed anhydride (*17*) or the cyclic anhydride (*16*) of diethylenetriaminepentaacetic acid (DTPA). Studies in patients with antibodies labeled with ^{111}In by either of these agents have often demonstrated more bone marrow and hepatic uptake than desirable. Uptake of only a small amount of radiometal by these tissues can be a substantial disadvantage. We
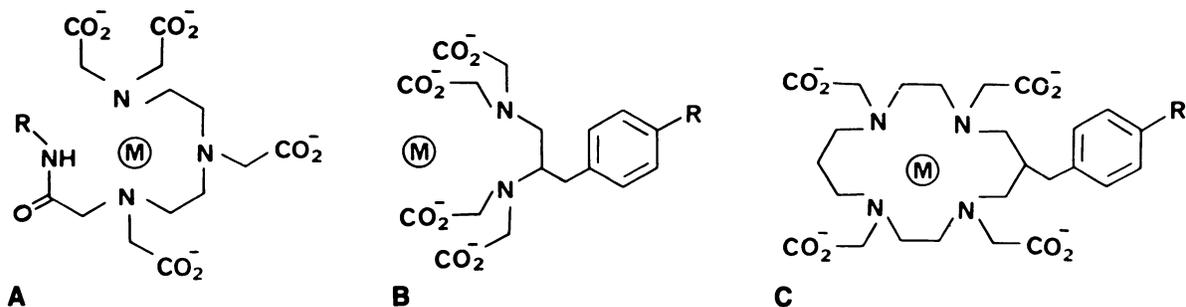
FIGURE 1
Structures of the three metal chelates are illustrated. A: DTPA, B: Benzyl EDTA, C: Benzyl-TETA. R represents the site of attachment to the antibody by way of a lysine amino group; when the chelates were studied without antibody attachment, R = butyl for DTPA, R = H for benzyl-EDTA, and R = NO_2 for benzyl-TETA.

have utilized not only the DTPA labeling group, but also new bifunctional chelate groups (Fig. 1) developed by Meares et al. (15,19) to radiolabel antibodies in order to compare their serum stability in vitro.

MATERIALS AND METHODS

General Analytic and Laboratory Techniques

Cellulose acetate electrophoresis (CAE) was performed with barbital buffer (0.05M, pH 8.6) for 30 min at 300 V.

Gel exclusion chromatography was performed with Sephadex G-25-80 columns (1.0 × 15 cm, V = 4.5 ml, V_t = 11.78 ml).

High performance liquid chromatography (HPLC) was performed at room temperature on a TSK 3000SW size exclusion column eluted with 0.1M sodium phosphate buffer, pH 7.4, containing 0.005% NaN_3 by weight. The flow rate was maintained at 1.0 ml per min, and uv absorbing substances were detected in the eluent by a flow-through detector operating with a 280 nm filter. Eluent fractions were collected and counted in an automatic scintillation well counter [NaI(Tl) crystal].

Ascending thin layer chromatography (TLC) was performed on silica-gel impregnated glass fiber sheets using a mobile phase of 10% (w/v) aqueous ammonium acetate: methanol, 1:1 (v/v). The mobile solvent front was allowed to run 10 cm per strip.

Gel centrifugation. Separation of antibody conjugates from excess chelates or radionuclides was achieved with a centrifuge procedure described by Pernefsky (20) and Meares et al. (15). One milliliter plastic syringe barrels were filled with a 60% slurry of Sephadex G-50 (fine) in the desired buffer and allowed to drain to a final gel volume of 0.6 ml. The gel-filled syringe was centrifuged in a tube for 2 min at ~100 g to a packed gel volume of ~0.4 ml. One hundred microliters (± 10 μl) of sample was applied to the top of the gel followed by centrifugation for 2 min as before. During centrifugation the effluent (~100 μl) was collected in a small conical plastic tube. This procedure allows rapid, quantitative separation of the sample components without significant dilution of the antibody.

Radiochemicals

$^{57}CoCl_2$ in 0.1 N HCl was obtained from ICN (sp. act. 7,000 Ci/g). $^{111}InCl_3$ had been purified by HCl elution from

an anion exchange resin (13) and was received as the dry solid. Copper-67 was provided by Los Alamos National Laboratory (21). The specific activity of the $^{67}CuCl_2$ (in 2N HCl) varied from 199 to 6,000 Ci/g Cu.*

Monoclonal Antibody (Lym-1)

An IgG_{2a} monoclonal antibody against B-cell lymphoma (22), was used for these studies. The antibody was purified from ascites fluid by a protein-A affinity column procedure developed and described by our laboratory (23).

Synthesis of Bifunctional Chelating Agents

Benzyl-EDTA (BEDTA) and its isothiocyanate (CITC) daughter were prepared according to Meares et al. (15). The reagent, CITC, was conjugated to the monoclonal antibody as described (15) by incubation of antibody (10 mg/ml) and CITC (1.5 mM) at pH 9 to 9.5 for 2 hr at 37°C. The antibody-chelate conjugate was isolated by gel-centrifugation into citrate or acetate buffer. P-Bromo-acetamidobenzyl-TETA and p-nitrobenzyl-TETA were prepared according to Moi et al. (19). Preparation of the ^{67}Cu-benzyl-TETA-antibody is described as follows.

DTPA-Lym-1

DTPA anhydride was prepared by the method of Eckelman et al. (16). All conjugation reactions between DTPA anhydride and Lym-1 were performed in 0.1M sodium phosphate buffer, pH 8. The reactions were allowed to proceed for 4–5 min at room temperature. The concentration of antibody exceeded 10 mg/ml (6.67 × $10^{-5}M$) and the initial concentration of the DTPA anhydride was 1 × $10^{-3}M$. The reaction was halted by subjecting the reaction mixture to the gel-centrifugation procedure.

Preparation of Radiochelates

All radiochelates were prepared at pH 5.0–5.5. Radiochelate formation was evaluated by TLC, where unchelated radiometal was readily distinguished from chelated radiometal (15).

Benzyl-EDTA and NO_2-benzyl-TETA. Benzyl-EDTA (BEDTA), the structural analog of CITC lacking the reactive isothiocyanate group, was labeled with cobalt-57 (^{57}Co), ^{111}In, and ^{67}Cu. NO_2-benzyl-TETA, the structural analog of bromoacetamidobenzyl-TETA was labeled with ^{67}Cu. These radiochelates were prepared at a chelator concentration of at least 1 × $10^{-4}M$. Because In^{3+} is less stable in aqueous solution, the ^{111}In chelate was prepared in 0.1M ammonium citrate,

TABLE 1
Percent Remaining as Intact Species When Analyzed by HPLC

	Day						
	0	1	2	3	5	7	10
[^{111}In]DTPA + serum	100	99.3	98.9	—	98.2	98.2	96.7
[^{111}In]DTPA-Lym-1 + serum	100	89.3	88.2	86.5	86.3	84.8	84.2
[^{111}In]BEDTA + serum	100	100	99.6	99.6	99.2	98.2	99.0
[^{111}In]BEDTA (CITC)-Lym-1 + serum	100	99.5	99.2	99.3	98.8	98.8	98.2

while the ^{67}Cu and ^{57}Co chelates were prepared in 0.1M ammonium (or sodium) acetate.

The chelators and radiometal were mixed at room temperature and analyzed by TLC (*13,15*). Control experiments were conducted in the same manner to ensure this TLC system would separate free copper from copper-chelate. When any unchelated radiometal remained, it was removed by passing the mixture through a 0.5 ml column of CHELEX-100 exchange resin.†

DTPA. The concentration of DTPA (*24*) during chelation was at least 2 × 10^{-4}M. The [^{111}In]DTPA chelate was prepared in 0.1M ammonium citrate while the ^{67}Cu and ^{57}Co chelates were prepared in 0.1M ammonium acetate. The mixtures of DTPA and radiometal were allowed to incubate for 1 hr at room temperature.

Radiolabeling of Lym-1 Conjugates

The addition of ^{67}Cu or ^{57}Co to the chelate or immunoglobulin chelate conjugate was carried out either in 0.1M sodium acetate or 0.1M ammonium acetate, pH 5.5–7.5. Indium-111 was added in 0.1M ammonium citrate pH 5.5–6.0.

CITC-Lym-1. When the excess CITC was removed from the CITC-Lym-1 conjugate by gel-centrifugation, the CITC-Lym-1 conjugate was concomitantly transferred to an acetate or citrate buffer depending on which radiometal was to be attached. The radiometal solution in the stated buffer was adjusted to pH 5–5.5 with 2N NaOH immediately before adding to the conjugate. The concentration of conjugate was maintained at least 1 × 10^{-5}M during radiolabeling. Fifteen minutes after the radiometal was mixed with the antibody-chelate conjugate, the solution was made 1 × 10^{-3}M in EDTA to scavenge any unchelated metal ions. Five minutes later, the product was isolated by gel-centrifugation.

The developmental work with CITC-Lym-1 was done with ^{57}Co due to its convenient physical T$_{1/2}$, gamma counting characteristics, and expected similarity with copper chelation chemistry.

The number of antibody-bound chelating groups was measured by TLC (*15*) using radiolabeled cobalt of known concen-

tration, and chelate/Ab ratios of 3:1-4:1 CITC/Ab were utilized.

Cobalt-57, ^{67}Cu, and ^{111}In-radiolabeled CITC-Lym-1 all were prepared in the same manner. In each case, the final product was transferred into 0.1M sodium phosphate, pH 7.5–8.0, during the final separation of antibody-bound radiometal from free radiochelate.

DTPA-Lym-1. DTPA-Lym-1 was radiolabeled with ^{57}Co, ^{67}Cu, or ^{111}In under identical conditions as described above for CITC-Lym-1. Separation techniques and analysis of chelate to antibody ratio were also identical, with 4:1 DTPA/Ab ratios.

Copper-67-benzyl-TETA-Lym-1. This was prepared by reacting the ^{67}Cu complex of bromoacetamidobenzyl-TETA with Lym-1 at 37°C, pH 9.5 (*19*). This yielded a [^{67}Cu]TETA/Ab ratio of 16:1. We subsequently developed a procedure for adding the metal to the Lym-1-benzyl-TETA conjugate (*19*).

Radioiodination of antibody. Lym-1 was radioiodinated for use in comparative serum stability studies. Radioiodination was performed by the chloramine-T method (*25*). Iodine-125 (NaI, 1300-1700 Ci/g I),‡ was substituted at the average level of 0.02-1.0 iodine atoms per antibody molecule using a 1:10 chloramine-T/antibody ratio in the reaction mixture for 3 min. The radioiodinated antibody was purified by gel chromatography. Evaluation of protein characteristics was obtained by cellulose acetate electrophoresis and high performance liquid chromatography (HPLC).

Preparation of human serum. Blood was collected from volunteers and allowed to clot for 1 hr at 37°C in a humidified incubator maintained at 5% CO_2, 95% air. The samples were centrifuged at 400 g and the serum was filtered through 0.22-μ syringe filters into sterile plastic culture tubes. Human serum prepared in this manner and stored at 4°C was used within 24 hr for in vitro serum stability studies described below.

In Vitro Serum Stability. Radiolabeled Lym-1, radiochelates of Benzyl-EDTA, NO_2-benzyl-TETA, and DTPA, and "free" radiometals were incubated for 10 days in human serum at 37°C in a humidified chamber maintained in 5% CO_2, 95% air (pH 7.0).

TABLE 2
Percent Remaining as Intact Species When Analyzed by HPLC

	Day						
	0	1	2	3	5	7	10
[^{57}Co]DTPA + serum	97.5	65.6	51.7	51.3	49.4	48.7	47.7
[^{57}Co]DTPA-Lym-1 + serum	95.1	56.5	50.9	54.1	53.7	52.8	52.8
[^{57}Co]BEDTA + serum	98.4	98.7	96.5	97.7	96.3	—	98.6
[^{57}Co]BEDTA (CITC)-Lym-1 + serum	100	—	94.6	94.3	90.1	—	92.2

Each sample consisted of 1.5–2.0 ml of serum in sterile culture tubes to which 100 μl of radioactive solution was added. Over the series of experiments, the amount of radio-labeled antibody added to serum varied from 80–800 μg (except for the [^{67}Cu]DTPA antibody where 2 mg was necessitated by the low ^{67}Cu specific activity). The serum concentration of radiochelates was below 10^{-5} molar; the concentration of excess chelator was $10^{-3}M$ for the Cu-DTPA samples, $10^{-5}M$ otherwise. In order to continue the study for 10 days with ^{67}Cu and ^{111}In, 10–50 μCi of total radioactivity was used for each serum sample.

Ten microliter aliquots of each serum mixture were analyzed by HPLC and CAE on Days 0, 1, 2, 3, 5, 7, and 10. Radioactivity profiles obtained from HPLC fractions were compared to the recorded ultraviolet absorbance profiles. The cellulose acetate electrophoresis strips were cut in half lengthwise, and one half was stained with Coomassie Brilliant Blue (0.25% in H$_2$O). The complementary side was counted for radioactivity distribution by scanning on a strip scanner or by cutting the strip into 2-mm sections and counting the sections in an automatic well-counter. The radioactivity distribution was compared to the spatial distribution of the stained serum protein bands and the stained bands of nonradioactive serum.

RESULTS

Serum Stability

The results of the serum stability study of radiochelates alone and radiochelates conjugated to the Lym-1 antibody are summarized in Tables 1–3. Figures 2–5 demonstrate the comparative radioactivity and serum protein profiles obtained from HPLC and cellulose acetate electrophoresis analysis. The radioiodinated immunoglobulin profile obtained by HPLC and cellulose acetate electrophoresis, demonstrated <5% loss of radiolabel and no evidence of degradation of the antibody molecule during the 10-day incubation period.

Under these conditions of labeling and incubation [^{111}In]CITC antibody is more stable than [^{111}In]DTPA antibody. Furthermore, the indium chelates (and indium chelates attached to antibodies) are more stable than their cobalt or copper counterparts. The cobalt chelates (and cobalt chelate-labeled antibodies) are in turn more stable than their ^{67}Cu-labeled counterparts. The copper chelates are extremely unstable in this
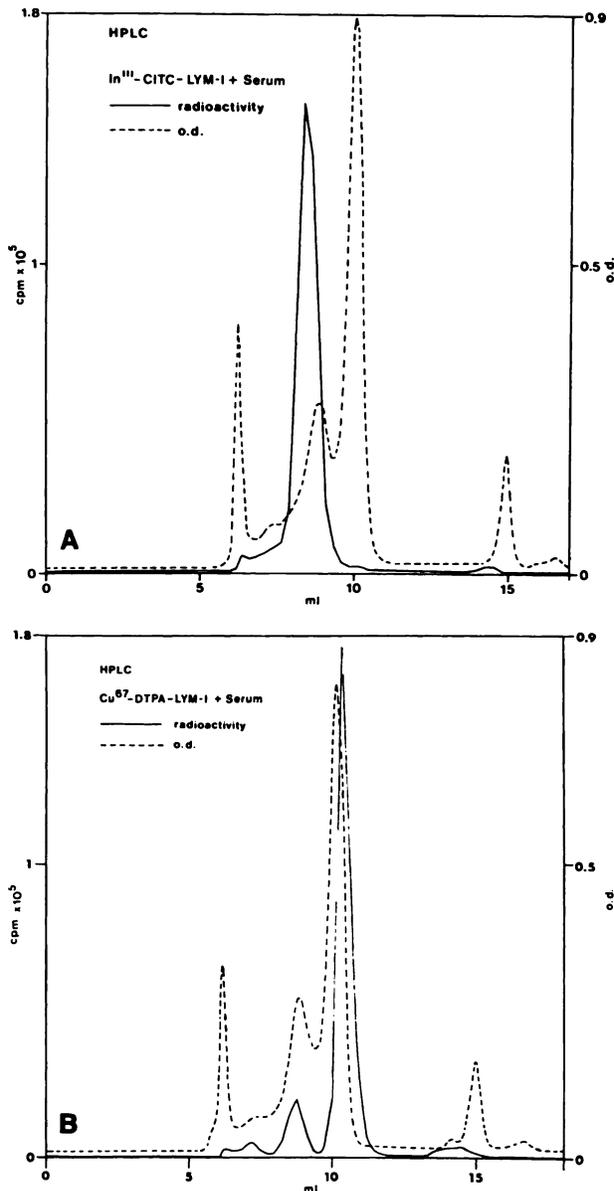

FIGURE 2

Ten microliter aliquots of each incubated serum mixture were serially evaluated over the 10-day study. HPLC-TSK-3000 chromatography yielded a column recovery of >90% of the injected radioactivity. A: [^{111}In]CITC (BEDTA)-Lym-1 incubated 1 day in serum demonstrated this serum profile by uv absorbance at 280 nm (OD). The radioactivity profile from this aliquot showed an unaltered radiometal antibody peak at 8.5 ml with <1% with transferrin at 10 ml or as free chelate (BEDTA) 14.5 ml. This profile remained constant for the entire 10-day period tested. B: [^{67}Cu]DTPA-Lym-1 after 1 day of incubation in serum had only 4–5% of the ^{67}Cu radioactivity on the 8.5-ml eluted antibody peak. The majority eluted with albumin 10.2 ml. This profile was duplicated by the [^{67}Cu]CITC (BEDTA)-Lym-1 stability study. Only TETA held the ^{67}Cu on the antibody when incubated in serum.

TABLE 3
Percent Remaining as Intact Species When Analyzed by HPLC

	Day						
	0	1	2	3	5	7	10
[^{67}Cu]DTPA + serum	100	23	31	35	33	31	31
[^{67}Cu]DTPA-Lym-1 + serum	100	4	5	6	5	5	5
[^{67}Cu]BEDTA + serum	95	14	17	18	—	18	24
[^{67}Cu]BEDTA (CITC)-Lym-1 + serum	95	5	6	4	5	7	7
[^{67}Cu]benzyl-TETA + serum	100	99	99	98	98	98	97
[^{67}Cu]benzyl-TETA-Lym-1	100	—	—	96	94	—	—

protein environment, except for the benzyl-TETA copper (± antibody) which, like the benzyl-EDTA indium, shows very little decomposition.

In-111-Citrate ☐
In-111-DTPA ▦
In-111-BEDTA ▥
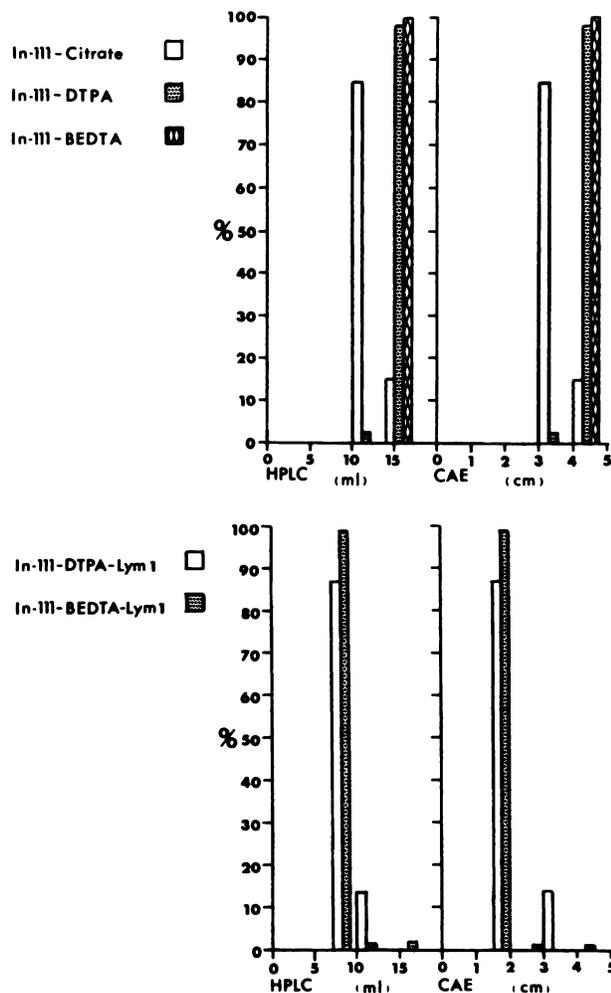
In-111-DTPA-Lym1 ☐
In-111-BEDTA-Lym1 ▦

FIGURE 3
Comparative radioactivity profiles from radiochelated and radiochelate-antibody mixtures which have been incubated in serum for 3 days. HPLC-TSK-3000 column chromatography and cellulose acetate electrophoresis (CAE) demonstrate the stability of ^{111}In-chelates and ^{111}In-chelated antibody. Stable radiolabeled chelates are present at 15–16 ml on HPLC, and 4-5 cm on CAE. Stable radiolabeled antibodies are at 8–10 ml on HPLC, and 1–2 cm on CAE. Albumin and transferrin associated radioactivity is seen at 10–12 ml on HPLC and 2.5–3.5 cm on CAE.

The loss of the radiolabel from each antibody-chelate conjugate was due to loss of the radiometal from the chelate and not the chelate moiety being lost from the protein, nor protein breakdown. A possible exception is that 0.7–2% of the BEDTA-In may have been released from the antibody during the 10-day period. Radiometals, when lost from their chelate attachment, were associated with serum proteins in a pattern particular to each radiometal (Figs. 2–5). When the indium is lost from the initial chelate, it migrates with transferrin on electrophoresis and elutes from HPLC TSK-3000 in the size range of transferrin. When ^{57}Co is lost from either chelate or antibody-chelate conjugate, it migrates on electrophoresis with many serum proteins

and separates on TSK with proteins of several molecular sizes. ^{67}Cu, leaving either simple chelating agents or antibody-chelate conjugates, is always observed in association with albumin (Figs. 2 and 5).

DISCUSSION

Preparation of radiopharmaceuticals from monoclonal antibodies is dependent upon development of labeling systems which can consistently attach the radionuclide in a predictable manner without significantly affecting the antibody molecule, and which can produce a final radioactive antibody with reliable stability in vivo. Recent interest in this goal has led to the development of a large number of different metallic reagents incorporating EDTA and DTPA groups. Previously
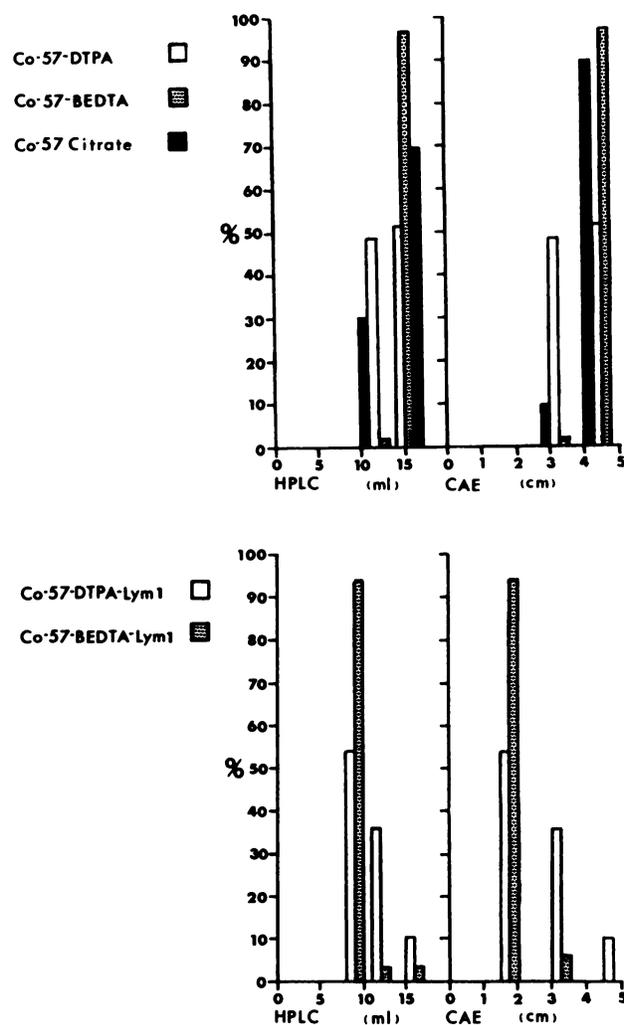

Co-57-DTPA ☐
Co-57-BEDTA ▦
Co-57 Citrate ■

Co-57-DTPA-Lym1 ☐
Co-57-BEDTA-Lym1 ▦

FIGURE 4
^{57}Co chelate and ^{57}Co antibody chelate radioactivity profiles from HPLC-TSK-3000 and cellulose acetate electrophoresis (CAE) after 3 days in serum incubation. ^{57}Co binds to various serum proteins when free, although albumin is the major carrier. [^{57}Co]BEDTA (CITC) is more stable in serum than [^{57}Co]DTPA.

Cu-67-DTPA ☐
Cu-67-BEDTA ▦
Cu-67-TETA ▨

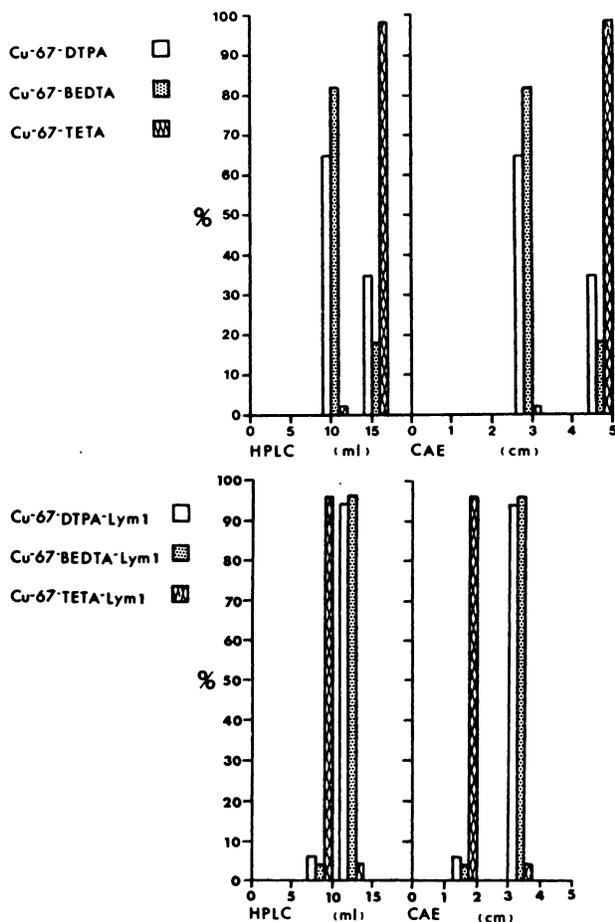Cu-67-DTPA-Lym I ☐
Cu-67-BEDTA-Lym I ▦
Cu-67-TETA-Lym I ▨

FIGURE 5
[67]Cu radioactivity profiles from HPLC-TSK-3000 and cellulose acetate electrophoresis (CAE) after 3 days of serum incubation, as [67]Cu-chelate or [67]Cu chelate antibody. [67]Cu binds to albumin in serum and only remains on the chelate and antibody chelate when the chelating agent is TETA.

published reactions for coupling chelating agents to proteins have included diazonium coupling (*12*), acylation with activated carboxyl groups (*7,11,14,16–18*), alkylation (*15*) and isothiocyanate coupling (*15*).

A previous comparison of various EDTA and DTPA indium chelates has demonstrated greater apparent stability of the benzyl EDTA indium complex in serum (*26*). Therefore, we have utilized the isothiocyanate derivative of benzyl-EDTA to study the stability of various radiometals in a system where the labeling conditions were well documented. The cyclic anhydride of DTPA, representing a clinically known bifunctional chelator, was assessed with the same radiometals for its comparative serum stability. When it became evident that copper was quickly lost from all those chelators, the benzyl-TETA chelators recently developed in our laboratory (*19*) were studied with [67]Cu. Indium-111 and [57]Co chelate with benzyl-TETA were not studied in serum. Benzyl-TETA readily forms chelates with [57]Co^{2+}, but not with [111]In^{3+}, in simple buffer solutions.

When dealing with the stability of metal chelates in solution, the equilibrium constants for the systems under study can provide useful information. Equilibrium constants can be used to predict the spontaneous direction of a reaction (e.g., whether a metal chelate will be thermodynamically stable in serum, or whether it will tend to dissociate). However, an equilibrium constant can reveal only the direction—not the *rate*—of a reaction. The properties of some of the copper chelates studied here provide a useful example. According to Lau and Sarkar (*27*), the logarithm of the apparent equilibrium constant for the binding of copper (II) to human serum albumin at physiologic pH is log K_{CuHSA} = 16.2. This number refers to the state of protonation of albumin at pH 7.4, and thus would be different at another pH. The logarithm of the corresponding equilibrium constant for the binding of copper (II) to EDTA at physiologic pH may be calculated by standard procedures from the thermodynamic equilibrium constant of copper (II)-EDTA (log K_s = 18.7) and the proton-binding constants for EDTA (log K_1 = 10.17; log K_2 = 6.11; log K_3 = 2.68; log K_4 = 2.00); the result is log K_{CuEDTA} = 15.9 at pH 7.4. Comparison of log K_{CuHSA} with log K_{CuEDTA} indicates that, at equilibrium, if the concentrations of EDTA and HSA are comparable, significant amounts of copper will be bound to each. The concentration of albumin in serum is ~0.5 × $10^{-3} M$ (*28*). Under realistic clinical conditions, the concentration of antibody-bound EDTA groups in serum certainly will be no greater than ~$10^{-7} M$; that is, a factor of at least 10^3–10^5 lower in concentration than albumin. At equilibrium under these conditions, practically all the copper will be bound to albumin; this will be true even if the equilibrium constants used above are in error by an overall factor of as much as 3 log units.

Of course, the studies described here did not employ unmodified EDTA, but rather an analog of EDTA with a benzyl group attached to a backbone carbon atom (Fig. 1). The equilibrium constants for these particular analogs have not been measured, but the literature contains numerous values for closely related compounds (*29*). These results indicate that the presence of a backbone substituent generally increases the stability of a metal-EDTA chelate by 1–2 log units, with little or no change in the proton-binding constants. For the $10^{-5} M$ benzyl-EDTA concentrations used in our experiments, it is clear that the position of equilibrium strongly favors albumin (Table 3).

For the macrocycle TETA, the equilibrium constants of Stetter et al. (*30*) indicate that log K_{CuTETA} = 12.6 at pH 7.4 after correction for protonation, so CuTETA is less stable thermodynamically than CuEDTA under physiological conditions. For the benzyl-substituted analogs studied here, the equilibrium constants for the relatively rigid macrocycle TETA are expected to be

affected less than the EDTA group, so that in any case copper-benzyl-TETA should be significantly less stable thermodynamically than copper-benzyl-EDTA. However, the data in Table 3 show that (in contrast to copper-benzyl-EDTA) copper-benzyl-TETA remains intact for several days in serum. This indicates that the loss of copper from either chelate is controlled by kinetic factors which depend on the chelate's structure. Such phenomena are familiar to inorganic chemists; in particular, macrocyclic structures such as that of copper-TETA are notable for slow kinetics. The interested reader is referred to the excellent review by Margerum et al. for more information (31).

Table 3 also shows that the loss of copper from DTPA to albumin occurs readily. For this chelate log K_{CuDTPA} = 17 at pH 7.4, making it much more stable thermodynamically than copper-benzyl-TETA. It is not known whether any of the original copper (II) becomes reduced to copper (I) during incubation in serum.

Kinetic control also applies to the loss of indium from benzyl-EDTA and DTPA chelates (26). Indium-transferrin has very high thermodynamic stability; published estimates of the first binding constant are in the range of log K_{InTf} = 30 ($32,33$). However, because of the complex properties of transferrin, its equilibrium constants have proven difficult to measure accurately; published estimates of the first iron-transferrin binding constant range from log K_{FeTf} = 20.7 (34) to log K_{FeTf} = 30.3 (35), and a strong correlation between the constants for iron and indium is expected (33). The corresponding log K_{InDTPA} = 24.6 at pH 7.4, after the required adjustment for proton binding to DTPA (27). As shown by Yeh et al. (26) under physiological conditions, with $2 \times 10^{-7}M$ DTPA present in the serum, the $10^{-5}M$ serum transferrin sites (28) are thermodynamically favored to pick up indium from the chelate. In serum, the rate of indium loss to transferrin depends on the structure of the chelate (26). The data in Table 1 show that indium is lost from benzyl-EDTA more slowly than from DTPA (log K_{InEDTA} = 22.1 at pH 7.4 when corrected for proton binding by EDTA; the stability of the benzyl-EDTA chelate is probably 1–2 log units higher). However, the $10^{-5}M$ concentrations of chelators used here were larger than the $2 \times 10^{-7}M$ used in (26), with the result that only a little indium is lost from the chelates.

Finally, Table 2 shows that cobalt is lost from its DTPA chelate (log K_{CoDTPA} = 14.8 at pH 7.4, corrected for protonation) much more rapidly than from the benzyl-EDTA chelate (log K_{CoEDTA} = 13.5 at pH 7.4, corrected for protonation; the stability of the benzyl-EDTA is probably 1–2 log units higher). It is not known whether the original cobalt (II) becomes oxidized to cobalt (III) during incubation in serum.

It would be preferable to study the behavior of each metal chelate in serum at trace concentrations and in

the absence of excess chelators. Because of the complexity of the study and the low specific activity of the available ^{67}Cu, this wasn't practical. However, conditions were chosen so that the DTPA samples, which have the highest thermodynamic stability constants, ($29,30,36$), also contained higher concentrations of free chelator than the others. This was done so that the slower loss of metal ions by the other chelators could be reliably interpreted as not being due to a more favorable equilibrium position.

The experiments described in this paper make it clear that the behavior of metal chelates in the complex biologic media encountered in vivo may be very difficult to predict. However, it appears that stable, practically useful chelators are available for the metals studied here, and there is a clear implication that the same chelators may successfully bind other metals as well.

NOTES

ACKNOWLEDGMENTS

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