A New Radiochemical Method to Determine the Stability Constants of Metal Chelates Attached to a Protein

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A new method was developed to determine the stability constants of bifunctional chelates of indium (In) coupled to a protein. This method utilizes the displacement reaction between an indium complex and ferric ion. By measuring the position equilibrium constant 'K' of this reaction and knowing the stability constant of the corresponding ferric chelate, the overall formation constant of the indium chelate can be determined. Human serum albumin (HSA) was conjugated with ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) using their cyclic dianhydrides. A new method was developed to couple triethylenetetraminehexaacetic acid (TTHA) with HSA using Woodward's Reagent K. The chelating agents coupled to HSA were complexed with indium-114m-(^{114m}In) labeled indium and purified by dialysis or microcentrifugation. The stability constants of these indium complexes were determined at physiologic pH using ferric chelate of nitrilotriacetic acid (Fe-NTA) as the source of ferric ion. No significant differences were found between the stability constants of the indium chelates conjugated to protein and those of unconjugated species.

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Macromolecules, such as human serum albumin (HSA), immunoglobulins, monoclonal antibodies and other proteins are easily radiolabeled with metal atoms by coupling them with bifunctional chelating agents, and followed by chelation with metal ions (*1–6*). The use of radiolabeled antibodies is based on the notion that they behave as magic bullets; that is, they bind only to those specific antigens against which they have been produced. In practice, however, when metal chelating groups such as diethylenetriaminepentaacetic acid (DTPA) are coupled to a monoclonal antibody that has been prepared against a tumor antigen and then radiolabeled with either indium-111 (^{111}In) or indium-113m (^{113m}In), only a fraction of this injected radiolabel is found to localize in the tumor site and most of the radiolabel appears in the liver and other organs (*7*). One possible explanation for this phenomenon is that the stability of the In-DTPA chelate could be different from that of the one protein bound. Indium forms very stable chelates with polyaminocarboxylic acids, to completion even at very low pH values, and it forms insoluble hydroxides also at low pH values (*8, 9*). These two factors make the determination of stability constant for these chelates extremely difficult. However, Schwarzenbach (*10*), determined the formation constant of the indium chelate of ethylenediaminetetraacetic acid (In-EDTA) by measuring the relative stability of two indium complexes using both redox potential and polarographic methods. Botari et al. (*11*) determined the stability constant of the indium chelate of DTPA (In-DTPA) by the redox potential method. However, the conditions that have to be used for performing stability measurements using these two techniques (redox and polarography), while very suitable for inorganic complexes, are not appropriate for chelates conjugated to proteins. Clearly, a mild method suitable for proteins and which will not affect their biologic integrity, is required. The present work aims to document a simple method to determine the stability constants of radiometal chelates conjugated to a protein and also of free chelates such as indium chelate of nitrilotriacetic acid (NTA) (In-NTA), EDTA (In-EDTA), DTPA (In-DTPA) and triethylenetetraminehexaacetic acid (TTHA) (In-TTHA).

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PRINCIPLE OF THE METHOD USED

The stability constants of indium chelates, such as In-NTA, In-EDTA, In-DTPA and In-TTHA, either in free form or in protein-conjugated form, can be determined using a displacement reaction between the In-chelate (In-Che) and ferric cation (Fe^{+3}), leading to the formation of the ferric chelate (Fe-Che) and tri-positive indium ion (In^{+3}) (*12, 13*). The equations representing the reactions of the 1:1 chelates are as follows:

$$\text{In-Chelate} + Fe^{+3} \leftrightarrow \text{Fe-Chelate} + In^{+3} \quad (1)$$

$$K = \frac{(\text{Fe-Che})(In^{+3})}{(\text{In-Che})(Fe^{+3})} = \frac{\beta_{(\text{Fe-Che})}}{\beta_{(\text{In-Che})}}, \quad (2)$$

where 'K' is the position equilibrium constant, $\beta_{(Fe\text{-}che)}$ is the stability constant of the Fe-Che and $B_{(In\text{-}che)}$ is the stability constant of the In-Che. If the constant 'K' can be determined experimentally, then the value of $B_{(In\text{-}che)}$ can be calculated when $\beta_{(Fe\text{-}che)}$ is known (*14*). The equilibrium concentrations of the various species are related by the law of mass balance principle (*14*). It follows from Equation 1, that the total concentration of indium or iron in equilibrium solution at any time is equal to the sum of the respective chelates and its free ions. Also, total metal chelates is the sum of In-Che and Fe-Che, which in turn is equal to the initial concentration of In-Che taken. Therefore, when the initial concentrations of indium and iron are known, and the molar concentration of In-Che (or free indium ions) is determined by analysis of the solution which has achieved equilibrium, then the molar concentration of free indium ions (or In-Che) and of free ferric ion and Fe-Che chelate can be calculated using mass balance equations (*14*) and hence 'K' can be determined.

For the study of protein-bound chelates (HSA-Che-In), Fe^{+3} is added in the form of ferric chelate of nitrilotriacetic acid (Fe-NTA) and the reaction is expected to proceed as follows:

HSA-Che-In

$$+ \text{Fe-NTA} \leftrightarrow \text{HSA-Che-Fe} + \text{In-NTA} \quad (3)$$

$$K = \frac{(\text{HSA-Che-Fe})(\text{In-NTA})}{(\text{HSA-Che-In})(\text{Fe-NTA})} = \frac{\beta_{(\text{HSA-Che-Fe})}}{\beta_{(\text{HSA-Che-In})}}. \quad (4)$$

In analyzing these data the stability constant of the HSA-Che-Fe is taken as that of the free Fe-Che (*15*), since no data are available for protein-bound metal chelated bifunctional complexes. This also assumes that the conjugation did not affect the stability constant of the ferric chelates. Naturally it is obvious that any differences in the values for the stability constants of the protein-bound and the free chelates may shed light on the modification of the chelating ability caused by conjugation to protein.

MATERIALS AND METHODS

All chemicals used, unless otherwise indicated, were of analytical grade, obtained from the following sources, and used without further purification: DTPA, TTHA, tetrasodium ethylenediaminetetraacetic acid (EDTA-Na4), cyclic DTPA anhydride (cDTPAA), Hepes buffer and HSA (essentially fatty acid and globulin free) (Sigma Chemical Co., St. Louis, Mo); indium chloride ($InCl_3$) anhydrous 99.999% pure, Woodward's Reagent K (WR), hydrochloric acid (HCl), sodium hydroxide (NaOH), and NTA (Aldrich Chemical Co. Milwaukee, WI); ferric chloride hexahydrate ($FeCl_3$), ferric nitrate (Mallinckrodt, Inc., St. Louis, Mo); ^{114m}In-chloride (gift from New England Nuclear Corp., Boston MA); cyclic EDTA dianhydride (cEDTAA) (gift from Dr. R. Schneider, SUNY Health Science Center, Syracuse, NY)

Preparation of the Chelates

Stock solutions of the chelating agents NTA, DTPA, and TTHA in 0.01 *M* concentration were prepared in deionized water (obtained by passing the demineralized water through a double column of chelex medium), neutralized to pH 7.0 with 1 *M* NaOH solution, after which the pH was reduced to a range of 3.3–4.0 by the addition of 1 *M* HCl. The EDTA-Na_4 was dissolved in the deionized water and the pH was adjusted to 3.3–4.0 with 1 *M* HCl. The pH measurements were made using the pH meter and a standard combination electrode (Beckman Model 3500, Beckman Instruments, Inc., Somerset NJ; Combination Electrode, Corning Co., Corning, NY). Concentrated solutions of $InCl_3$ (0.03 *M*, pH 2.4) and $FeCl_3$ (0.1 *M*, pH 1.3) were prepared using deionized water and dilute HCl. Fe-NTA of known concentration was prepared by dissolving ferric nitrate in NTA solution of equal molarity. The pH was adjusted slowly with the addition of dilute NaOH. The preparation of this stock solution has to be carried out slowly, and may take several days.

To a known amount (0.001–0.002 mmol) of the chelating agent (NTA, EDTA, DTPA, or TTHA) a small volume (0.2 ml) of radioactive ^{114m}In-chloride solution (1–2 mCi in 0.05 *M* HCl) was added. This mixture was vortexed well for 2 min and subsequently incubated for 10 min at room temperature. Then an equimolar amount of $InCl_3$ was added, vortexed well and incubated for at least 10 min to insure complete chelate formation.

Metal and Ligand Ratio

It was observed that TTHA is capable of forming polynuclear complexes with indium ions. To determine the effect of metal, ligand ratios, four different compositions of TTHA and In in the ratios of 1:1, 1:2, 1:3, and 1:4, were prepared and the ratio of indium bound to total indium was determined by paper chromatographic analysis as described below.

HSA-TTHA Conjugation and Labeling with ^{114m}In and Purification: Woodward's Reagent K Method

A new method was developed for coupling TTHA with HSA using Woodward's Reagent K (WR) (N-ethyl-5-phenylisoxazolium-3'-sulfonate) (*16–18*). WR is a good peptide-bond forming agent under a wide range of pH and temperatures, especially so in neutral and alkaline conditions (Fig. 1).

In a 50-ml beaker, 400 mg HSA (0.006 mmol) was dissolved in 10 ml of hepes buffer 0.1 *M* at pH 7.0. A solution of 30 mg TTHA-Na (0.06 mmol) in 2 ml of water at pH 8.0 was prepared in a 50-ml beaker. Forty-six milligrams (0.18 mmol) of WR was dissolved in 1.0 ml of water in a test tube. This solution was acidic and pH was <3.5. This freshly made WR solution was added to the TTHA-Na solution and mixed together for ~4 min. After adding the above HSA solution to this activated TTHA solution and upon mixing, the pH went down to 5.5. The pH was raised to 7.5 during the mixing by dropwise addition of 0.5 *M* NaOH (pH meter Beckman 3500, combination electrode, Corning). Then the mixture was continuously stirred for 6 hr and then left overnight at room temperature (22°C). The final volume was adjusted to 20 ml with the addition of hepes buffer (pH 7.0).

Pure conjugated sample was obtained by dialyzing the reaction mixture against one liter of water for 6 days at room temperature (water was changed once everyday) to remove unreacted TTHA. After dialysis the sample was labeled stoi-

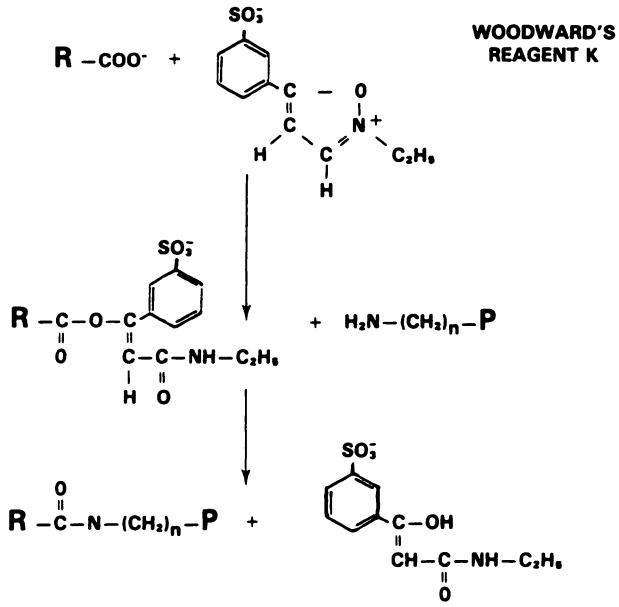

FIGURE 1
Reaction scheme of Woodward's Reagent K in protein conjugation of carboxylic compounds.

chiometrically with ^{114m}In-indium citrate at pH 6.3 and incubated at room temperature for 2 hr before analysis. A portion of this HSA-TTHA-In chelation mixture was dialysed against water for 5 days at 4°C to remove any unreacted ^{114m}In-In-citrate.

P6DG Analysis

Purity of the conjugated and indium-chelated protein was checked by P6DG gel chromatography using 30-cm × 1-cm column and P6DG desalting gel (BioRad, Richmond, CA) and 0.1 *M* hepes buffer at pH 7 as eluant at a flow rate of 0.3 ml/min. The gel column was connected in series with a pump (peristaltic), an ultraviolet (UV) (280 nm) detector with its recorder and a radioactivity (RA) detector (NaI(Tl) flow-through counter) with its recorder. A volume of 0.1–0.4 ml sample was used.

Ultrafiltration

Since complete purification of the protein chelate (HSA-TTHA-In) was not achieved by dialysis alone, as determined by chromatographic analysis, a final purification was carried out by ultrafiltration using centricon-30 (M. wt. cutoff 30,000) tubes (Amicon Corporation, Bedford, MA) and microcentrifuging the sample at 3,000 g for an hour. The residue was washed three times with 0.1 *M* hepes buffer at pH 7.0 and centrifuged as before. Pure sample was collected completely, with the addition of 0.1 *M* hepes buffer at pH 7. The microcentrifuged sample was stored at 4°C. This sample was checked for purity by P6DG analysis in comparison with standard radioiodinated (^{125}I) HSA (RIHSA).

Conjugation of Prechelated (Labeled) IN-TTHA to HSA Using the WR Method

In a 50-ml beaker, 80 mg HSA (0.0012 mmol) was dissolved in 2 ml hepes buffer pH 7.5. Labeled In-TTHA (0.08 mmol) with ^{114m}In (pH 8.0) was prepared as follows: 0.08 mmol indium chloride in 2 ml solution (pH 2.4) was mixed well with 0.5 ml (0.5 mCi) ^{114m}In in 0.5 *M* HCl. To the prepared labeled indium chloride solution in a 50-ml beaker, 0.08 mmol TTHA-Na salt solution (pH 3.5) in 2-ml volume was added, mixed well, and incubated for 10 min. The pH of this solution was found to be <3.0 and was raised to pH 8.0 by dropwise addition of 0.5 *M* NaOH.

Sixty-two milligrams (0.24 mmol) WR was dissolved in 2 ml chelex water and added to the labeled In-TTHA solution at pH 8.0. After 4 min, this In-TTHA-WR solution was added slowly to the HSA solution with stirring, and the pH was adjusted to 7.5 by adding 0.5 *M* NaOH dropwise. The conjugation mixture was stirred for 6 hr, made up to 10 ml by the addition of hepes buffer, and analyzed by P6DG gel chromatography to test for conjugation. To purify this sample, the protein chelate was ultrafiltered by microcentrifugation. The purity of this microcentrifuged sample was tested by P6DG gel chromatography and compared with that of RIHSA.

Coupling of EDTA and DTPA to HSA Labeling with ^{114m}In and Purification

Both EDTA and DTPA conjugates of HSA were prepared by methods similar to those described by Hnatowich et al. (*2*) using cyclic EDTA dianhydride and cyclic DTPA anhydride respectively. Briefly, in both cases the solid HSA (0.0003 *M*) and the anhydride (0.003 *M*) in 1:10 molar ratio was added to hepes buffer, 10 ml (pH 7.0) and mixed well and left at room temperature (22°C) for 24 hr (cEDTA dissolves slowly).

The chelation was carried out by adding ^{114m}In-indium citrate (pH 6.3) in excess (1:2 ratio of chelon to indium) to EDTA, DTPA coupled HSA solution. The solution was kept overnight, after mixing well, to insure complete chelation.

The HSA-EDTA-In and HSA-DTPA-In mixtures were purified by either centrifugal ultrafiltration using centricon 10 tubes or by exhaustive dialysis. The purity of the chelated protein sample was checked by P6DG gel filtration chromatography in comparison with standard ^{125}I-labeled HSA. Pure samples were stored at 4°C.

Determination of the Number of Chelating Groups Attached to HSA Molecule

The number of EDTA, DTPA, or TTHA groups attached to HSA was determined by measuring the molar amount of indium remaining in the pure sample (obtained by microcentrifuging the respective conjugated and indium-chelated HSA sample) using the known specific activity and concentration of carrier indium of the original chelation mixture. By comparing the amount of indium with that of protein present in the respective conjugated sample, the number of chelating groups per HSA was determined, assuming that indium and the chelating agents from 1:1 chelates. The protein concentration was determined by considering the amount of protein

taken for conjugation and the dilutions involved at different stages. The number of EDTA, DTPA, and TTHA groups per HSA molecule were found to be 8.5, 7.8, and 3.9, respectively. For a preformed TTHA-In conjugation with HSA, the number of groups was 1.5.

Equilibrium Study and Analysis

Indium Chelates: To the desired ^{114m}In-labeled In-Che solution of known amount, prepared as described above, an equimolar quantity of $FeCl_3$ was added. The solutions were mixed well for 2 min and were allowed to reach equilibrium at the desired temperature, 20 ± 1°C. Samples of this equilibrium mixture were analyzed at various intervals, as described below, using paper chromatography.

Paper Chromatography. The equilibrium mixtures of free chelates were analyzed by paper chromatography using Whatman 31ET paper and 0.1 *M* ammonium acetate (Fisher Scientific Co., Los Angeles, CA) solution (pH 7.4) as the solvent. Paper strips spotted with the equilibrium mixtures were air dried, developed individually in the solvent, and dried well. The dry paper strips were cut into 1-cm pieces and counted in a 2-in. NaI (Tl) well-counter. The displaced In^{+3} ion stays at the origin as indium hydroxide, while the In-Che migrates to the solvent front. Suitable standards of $InCl_3$, In-DTPA, etc. were analyzed for comparison.

The equilibrium concentrations of In^{+3}, In-Che, Fe^{+3}, and Fe-Che were calculated using the analytical data of indium ion or In-Che and initial concentration of indium and iron.

HSA-Chelate-In. Pure, conjugated and indium-labeled protein chelate (HSA-DTPA-In, HSA-TTHA-In, and HSA-EDTA-In) in 0.1 *M* hepes buffer and pH 7.4 were prepared and their indium content determined as described above. To a known amount of protein-coupled In-Che solution in 0.10 *M* NaCl, Fe-NTA (1:1 complex) pH 7.0, was added in slight excess (In and Fe in ratio of 1:2), mixed well by vortexing for 1–2 min and kept for equilibration at 20 ± 1°C. The pH of this mixture was measured at the end of the experiment (pH meter, Beckman Phaser 1, Microelectrode). All the equilibrium studies were carried out at pH 7.4.

Instant Thin Layer Chromatography (ITLC). HSA-chelate equilibration mixtures were sampled at various times and analyzed by an ITLC method. ITLC-SG (Gelman Sciences Inc., Ann Arbor, MI) and (1:1, V/V) methanol and 10% ammonium acetate solvent system was employed. The dried ITLC strips were cut into 1-cm pieces and counted in a 2-in. NaI (Tl) well-counter. HSA-Che-In stays at the origin while displaced indium, in the form of In-NTA, moves towards the solvent front away from the origin.

In order to quantitate the HSA-Che-In staying at the origin in the RA profile of equilibrium sample analysis, an ITLC control was run using HSA-Che-In without any Fe-NTA added to it. These two RA distribution profiles of ITLC (Figs. 6 and 7) were compared to identify the exact location where the HSA-Che-In peak ends (near the origin) and the other one, In-NTA, starts. Appropriate precautions were taken to adjust for overlapping of peaks.

The kinetics of equilibrium reactions for both free chelates and protein chelates were followed and the corresponding log β values were determined.

RESULTS

Stability Constant Determination of Free Chelates of Indium

The radioactivity profiles of In^{+3} ion (as indium hydroxide) and indium chelates by paper chromatographic analysis of the control and equilibrium samples of 1:1 mixtures of In-Che and ferric ion (chloride) are shown in Figures 2 and 3, respectively. From the distribution of the radioactivity between the origin (displaced indium) and solvent front (metal chelate) and the known concentrations of indium and iron in the original mixture, and using the law of mass balance principle in Equation 1, the equilibrium concentrations of all species were determined and, hence, the displacement constant 'K' in Equation 2 were calculated. Knowing the 'K' values and the logarithmic stability constants (log β) of ferric chelates (Fe-NTA: 16.3, Fe-EDTA: 25.0, Fe-DTPA: 28.7 and Fe-TTHA: 26.8) (*15*), the β values for In-Che were calculated as described above. The log β values for indium chelates of NTA, EDTA, DTPA and TTHA are given in Table 1. When known, the

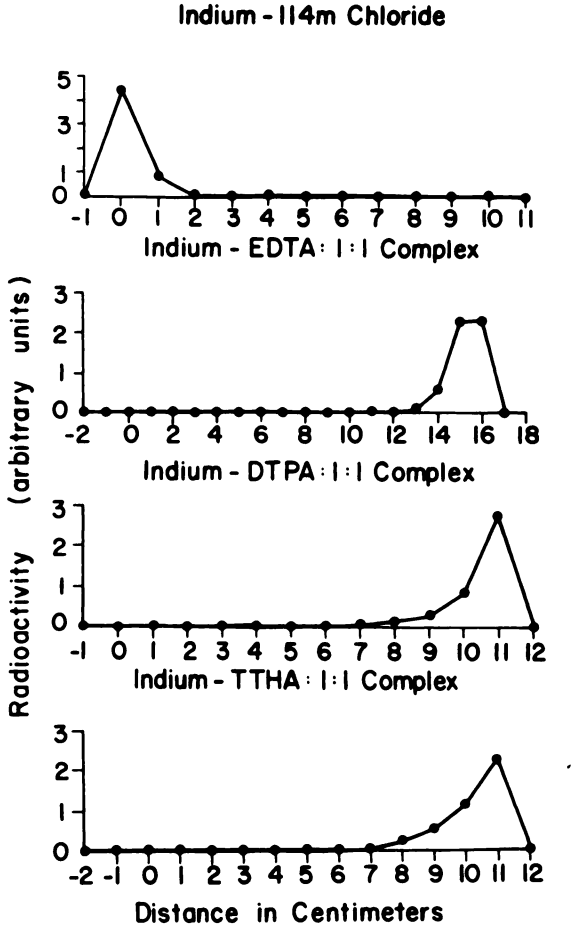

FIGURE 2
The radioactivity profiles of ^{114m}In-chloride and (1:1) stoichiometric indium chelates. Paper chromatographic analysis was carried out using Whatman 31 ET paper and 0.1 *M* ammonium acetate, pH 7.4, as solvent.

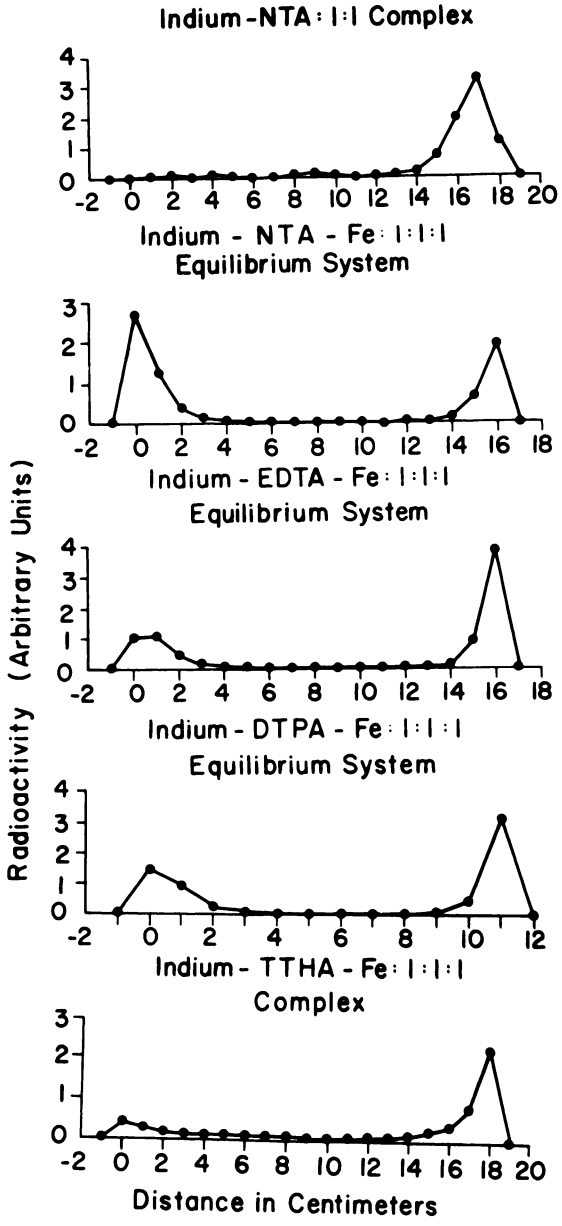

FIGURE 3
Radioactivity profiles of equilibrium samples of indium chelates containing ferric ions as displacing agent in 1:1 ratio in paper chromatography. Chromatographic conditions were same as that described for standards (see Fig. 2.) The equilibration time before analysis for NTA and EDTA was 72 hr and 24 hr for DTPA and TTHA (room temperature 22° C).

literature values are also included (*15*, *19*). The maximum metal-to-ligand ratio which can be attained in In-TTHA complex is shown in Figure 4.

Preparation of HSA-Coupled, Indium-Complexed Bifunctional Chelates

Combined radioactivity (RA) and ultraviolet (UV) profiles of HSA-coupled bifunctional chelates of indium before and after purification were obtained from P6DG gel chromatography.

Combined UV and RA chromatograms of the conjugation mixture of HSA and TTHA at different levels of purification are shown in Figure 5A-D.

TABLE 1
Stability Constants of Indium Chelates*

Chelates (n)	Log β	Log β (literature value)
In-NTA (9)	15.9 ± 0.19 at 22°C	16.9, 15.9
In-EDTA (8)	25.8 ± 0.21 at 20°C	25.3, 25.3
In-DTPA (6)	28.5 ± 0.08 at 20°C	29.0, 28.4
In-TTHA (9)	27.9 ± 0.18 at 20°C	not available

n = number of separate experiments.
* Equilibration time: 96 hr.

Stability Constants Determination of Protein-Coupled, Indium-Complexed Bifunctional Chelates

The radioactivity profiles of ^{114m}In with carrier indium in the control samples and equilibrium samples (1:2 mixture of HSA-coupled indium-complexed bifunctional chelate and Fe-NTA) on ITLC analysis are shown in Figures 6 and 7, respectively. Control sample analyses (Fig. 6) were used to determine the cutoff point which identifies the extension of protein peak in the ITLC profile (Fig. 7) of equilibrium sample. In each strip of ITLC analysis, the radioactivity not associated with the HSA-Che-In peak is assumed to be that of In-NTA. Trailing of In-NTA was observed in all equilibrium sample analyses. Appropriate precautions were taken to account for the peaks overlap. From the distribution of ^{114m}In activity between the origin (the undisplaced indium in protein complex) and the solvent front (displaced indium from the protein complex by Fe-NTA as In-NTA) on ITLC analysis and knowing the initial concentrations of indium and Fe-NTA in the original mixture, and using law of mass balance principle in Equation 3, the equilibrium concentrations of all the species were determined. From these values the position equilibrium constant 'K' in Equation 4 was calculated as described above. Knowing the 'K' values and β values for the protein-coupled Fe-Che, the log β value for Fe-Che used in this calculation are as follows: NTA: 16.3; EDTA: 25.0, DTPA: 28.7; TTHA: 26.8 (*15*). β values for HSA-Che-Fe are assumed to be the same as those of simple ferric chelates), β values for

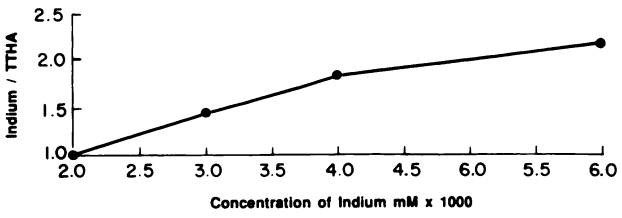

FIGURE 4
The effect of indium concentration on metal to ligand ratio in In-TTHA complex.

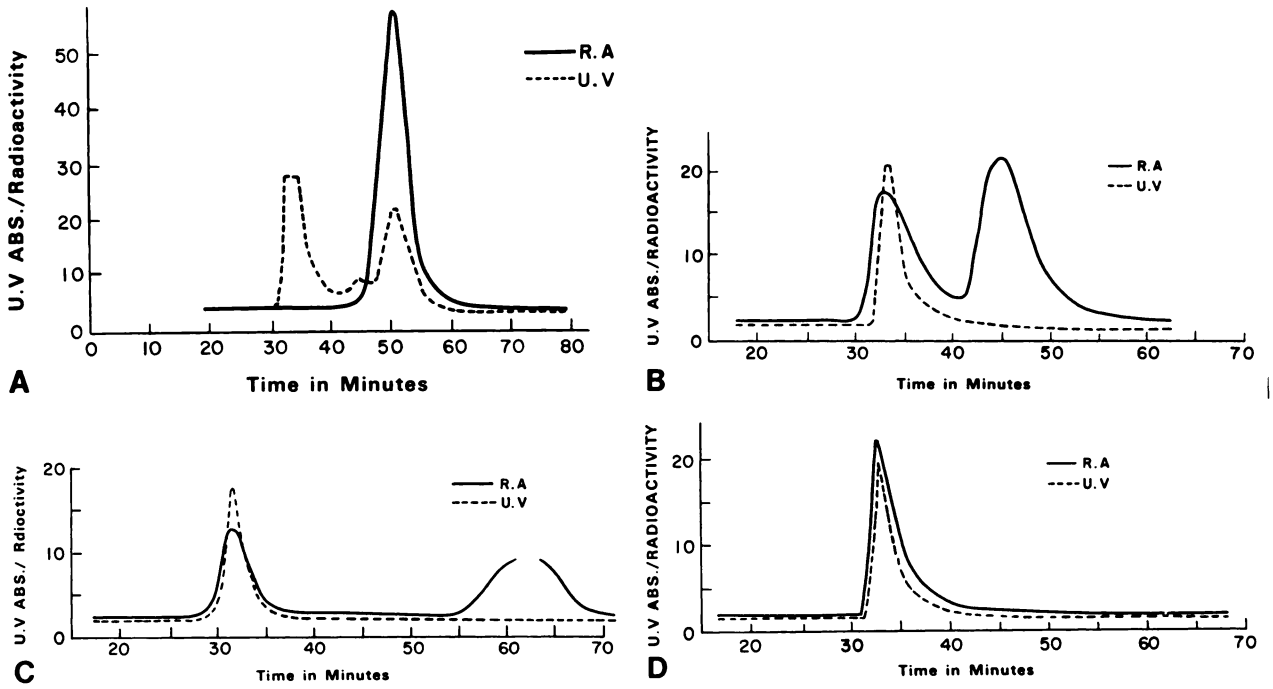

FIGURE 5

(A) Combined U.V. and R.A. elution profiles of conjugated, undialysed (to remove unbound TTHA) and labeled mixture of HSA-TTHA and ^{114m}In-In-citrate. Total activity is seen as associated complex (protein conjugated and nonconjugated associated with indium and indium chelates). (B) Combined U.V. and R.A. elution profile of HSA-TTHA dialyzed to remove unbound TTHA and then labeled with ^{114m}In-In-citrate. Sample was not purified after labeling. The first peak corresponds to conjugated protein. The second peak shows the presence of In-TTHA and indium citrate as impurities. (C) Combined U.V. and R.A. elution profile of double dialyzed (before and after labeling) HSA-TTHA-In (^{114m}In) sample. The second radioactivity peak shows the presence of indium, which was confirmed by microcentrifugation of the sample followed by the ITLC analysis of the filtrate. (D) Combined U.V. and R.A. elution profile of HSA-TTHA-In after purification. This sample was obtained by microcentrifugating (Centricon 30) the double dialyzed sample. This was used in stability constant determination.

protein-coupled indium-complexed bifunctional chelates were calculated. A minimum of three ITLC analyses were carried out for each sample at each interval and log β value was calculated in each case and an average of these values was taken. The log β values for the protein complexes are given in Table 2.

The kinetics of the various equilibrium reactions of the protein-coupled indium-complexed bifunctional chelates and of the free chelates are compared in Figure 8.

DISCUSSION

Very little work had been reported on equilibrium studies of In-Che of polyaminopolycarboxylic acids such as EDTA, DTPA, and TTHA. One recent paper had investigated the chelates of aluminum, gallium, and indium (*20*). Such equilibrium studies, and, hence, the determination of the formation constant of In-Che (and of other metals in the same group) had been hindered by the fact that they form very stable, inert complexes which do not attain an equilibrium, but where the reaction goes to completion, even at a very low pH. In addition, indium forms hydroxides also at low pH (*8*, *9*). While some data is available for indium complexes of NTA, EDTA and DTPA (*15*), nothing is available for TTHA. Khan et al. (*20*), in their recent work, reported that they were unable to determine the stability constant of 1:1 complexes of In-TTHA by potentiometric titration, because of the rapid completion of the formation reaction. In addition, methods like redox potential and polarography (*10*), which are the "standard" methods for determining stability constants of indium chelates, cannot be used for chelates conjugated to proteins in as much as they require pH conditions that are incompatible with the conformational integrity of proteins. Hence, a simple procedure using mild conditions needs to be developed to carry out such measurements with indium chelates conjugated to proteins used as radiopharmaceuticals. The method presented here is simple and less time-consuming for determining the stability constants of In-NTA, In-EDTA, In-DTPA, and In-TTHA as free chelates and also as protein-coupled chelates.

In the development of this radiochemical method, a simple displacement principle was employed as shown in Equation 1. Astakhov et al. (*21*) used this principle and radiochemical analysis in the determination of the stability constant of Ca-EDTA, Sr-EDTA, Ba-EDTA, etc., using ^{45}Ca. But their reported values and those of

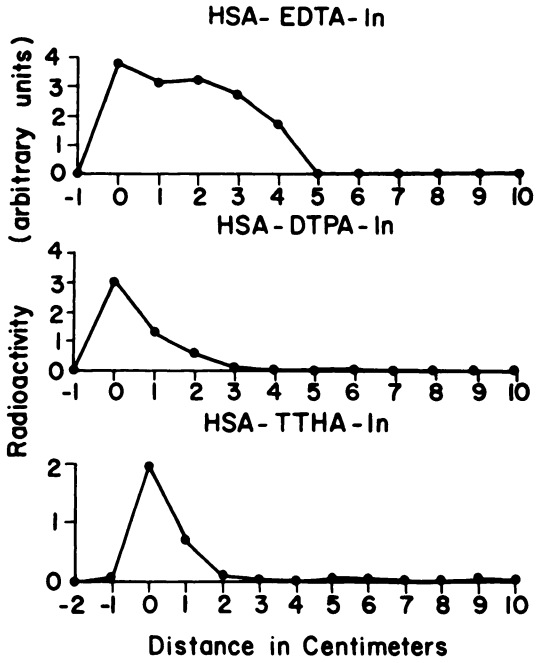

FIGURE 6
Radioactivity profiles of HSA-coupled indium chelates as seen in ITLC analysis. ITLC-SG support and 10% ammonium acetate and methanol (1:1 V/V) as solvent were used. The undisplaced indium in the protein complex stays at the origin.

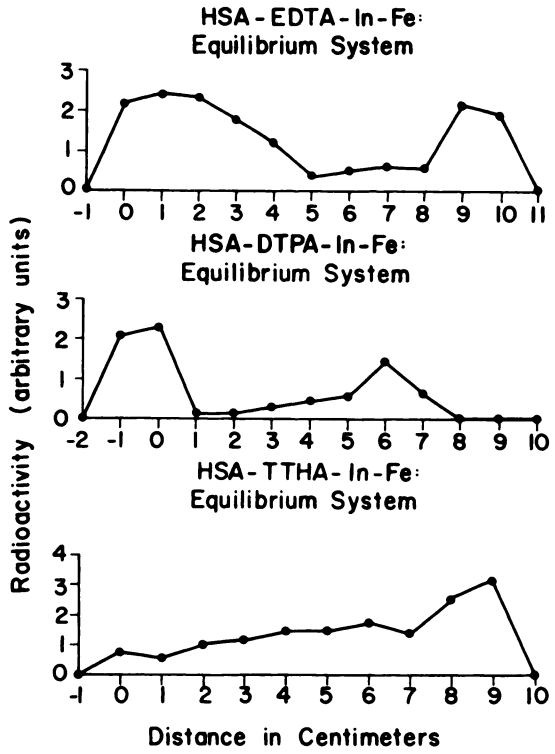

FIGURE 7
Radioactivity profiles of indium in equilibrium samples of HSA-coupled indium chelates containing Fe-NTA in ITLC analysis. The conditions are same as that described in Figure 6. Two separate peaks correspond to the undisplaced indium in HSA-chelate-In at origin and displaced In as In-NTA moving towards the solvent front.

others do not agree well. Betts et al. (*22*) used a similar technique in the study of the kinetics and thermodynamic properties of complex ions of the rare earths. They report that the stability constants of such EDTA complexes are in close agreement with the literature values. Unfortunately, their procedure and their calculations are lengthy and tedious.

In the present work we attempted to make the procedures and the calculations as simple as possible without sacrificing the accuracy of the data. Since the reaction that is to be measured is between the In-Che and the ferric ions (complexed to a weaker chelating agent), and it does not involve H^+ ions, it was possible to avoid the addition of buffer solution. Hence, and under the conditions used in this work, the need for additional inert salts also does not exist. Therefore, in the equilibrium analysis of free chelates, no buffers or inert salts had to be added to keep either the pH or the ionic strength constant.

The stability constants of the In-EDTA chelate and its analogs can be determined in a very short time, between 2 to 4 hr. The stability constants of the protein-conjugated indium-labeled chelates can be determined (see Fig. 8) in a similar manner. Calculations have been simplified significantly, especially when compared to the procedures previously available (*14*, *22*). From the results obtained (Table 1), it can be seen that the log β values 25.8, 28.5 for In-EDTA and In-DTPA, respectively, in this method agree well with those values 25.3 for In-EDTA and 29.0, 28.4 for In-DTPA reported in the literature (*15*, *19*). Because of this agreement between both values, this present method was employed to determine the stability constants of In-TTHA and protein-coupled indium chelates.

The conjugation of TTHA to HSA was carried out using WR, a good peptide bond-forming reagent under a wide range of pH and temperatures. WR forms an ester with the carboxylic group of the acid and, hence,

TABLE 2
The Formation Constant of Protein-Coupled Indium-Chelated Bifunctional Complexes

Temperature: 20 ± 1°C Equilibration time: 96 hr Ionic strength: 0.1 *M* NaCl		
n	Complex	Log β
8	HSA-In-DTPA*	29.3 ± 0.11
9	HSA-In-DTPA†	28.3 ± 0.03
14	HSA-In-TTHA	26.0 ± 0.08
10	HSA-In-EDTA	25.9 ± 0.13

*† Refers to the number of DTPA groups, 7.8 and 16.6, respectively.
n is the number of separate experiments.

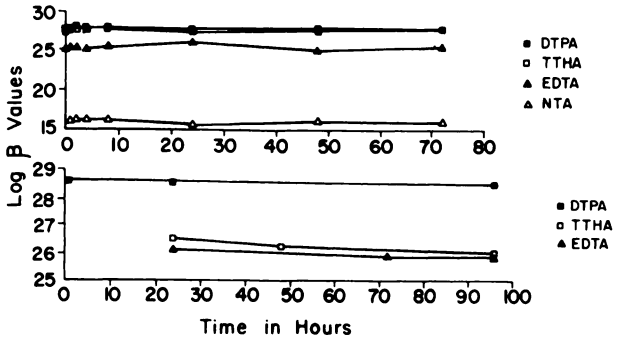

FIGURE 8
(Top) Kinetics of attaining equilibrium in free indium chelate after mixing with equimolar amount of ferric chloride solution. Corresponding log β values were determined. Equilibrium was reached in <30 min in all cases. (Bottom) Kinetics of the equilibrium reaction of HSA coupled to indium chelates. The kinetics of attaining equilibrium in HSA coupled indium chelates after mixing with Fe-NTA. While HSA-DTPA-In reaches equilibrium in 2 hr, EDTA and TTHA complexes required ~3 days and 4 days, respectively. The corresponding log β values were calculated.

makes it an activated compound. So the activation time becomes an important factor during conjugation. After the addition of the activated acid to the protein, it is essential to maintain the pH slightly alkaline in order to avoid the interference from WR byproduct generated during conjugation.

Conjugation of TTHA to protein by WR method eliminates the use of large quantities of the protein, the TTHA or the WR. HSA, TTHA, and WR reacted in the molar ratio of 1:10:30, results in approximately four groups of TTHA per HSA molecule. Due to the very high reactivity of this multidentate ligand, it seems the chelon forms some weak bondings (nonspecific) with protein molecules in addition to conjugation, which hinders the metal chelation of the coupled chelon. The conjugated TTHA is unable to chelate readily with metal ions (Fig. 5A.) Because of this, the conjugation mixture must be purified before chelation with metal ions. The gel chromatography profiles of conjugated, dialyzed and chelated mixture, Figure 5B, shows the presence of conjugated protein as a separate peak and In-TTHA and indium citrate as another peak. While dialysis can be used to purify the HSA-TTHA conjugate mixture, it was found that dialysis alone was not enough to purify the chelated sample. In this case, dialysis is unable to remove all the metal ions that have been bound weakly to multidentate chelons which comes off during P6DG gel chromatography (Fig. 5C.). Microcentrifuging the sample shown in Figure 5C offers the pure sample (Figure 5D), which elutes at the same time interval as that of HSA-EDTA-In and HSA-DTPA-In showing the sample is mononuclear.

In the ITLC, analyses of the equilibrium solution of protein conjugated and labeled indium complexed sample and Fe-NTA at various times after mixing, it was noticed that there was a steady increase of In-NTA formation (expected from the reaction principle and the mechanism of reaction) until the equilibrium was attained and thereafter the concentration of In-NTA remained constant. But there was no increase in indium concentration at the origin in the ITLC analyses observed at any time. These results show that indium is present in the equilibrium solution either as indium-chelated protein complex or as In-NTA. Therefore, the chances for the existence of free indium ion in equilibrium solution under the experimental conditions is nil or minimal. It follows that the indium hydroxide formation at the origin in ITLC analyses is also nil or minimal. After equilibrium, the concentration of In-NTA remained steady over a long period of time (After 18 days, HSA-TTHA-In equilibrium sample on analysis showed the same amount of In-NTA as that found after the sample attained equilibrium in 4 days.) This shows that In-NTA formed does not undergo hydrolysis under the experimental conditions employed in this method.

From Tables 1 and 2, it can be seen that the protein-bound indium-labeled bifunctional chelate is more stable than the free chelates in the case of DTPA and EDTA. But, in the case of TTHA, the stability constant of HSA-TTHA-In is lower than that of the free chelate.

Also from the data, it can be inferred that the indium ion in the complex with high stability constant is replaceable by metallic cations, such as ferric ion, in both acidic and neutral conditions. These results suggest that it may be possible that indium complexes of immunoglobulins and monoclonal antibodies (labeled with ^{111}In through bifunctional chelating agents) could be susceptible to displacement by ferric ions in vivo. Since ferric ions are available for binding to transferrin, under biologic conditions, they may also be available to interact with protein-coupled chelates, even when labeled with indium. This metal transchelation will not, however, occur with ferric ions already bound to transferrin, because of the higher stability constant of the iron binding to that protein. This has been observed under in-vitro conditions (*23*, *24*). Based on the results of this investigation, it can be postulated that high concentrations of indium found in the liver after injection of indium-labeled monoclonal antibodies may be due to the localization of indium displaced by ferric ions in addition to the natural localization of labeled monoclonal antibodies. When ferric ion displaces indium from its chelate, the released indium may form colloids (indium hydroxide) at physiologic pH and localize in the reticuloendothelial system. However further experimental studies are necessary to prove this contention.

In the present study, HSA was used as a model protein for conjugation with bifunctional chelating agents EDTA, DTPA, and TTHA in order to determine the overall stability constants of indium chelates when the chelate is attached to the protein through an amide

bond. Although the principle involved in determining the stability constants in this method may be equally applicable to other proteins, such as immunoglobulins and monoclonal antibodies, or different metal ions of compatible nature, the analytical procedures to be used may have to be modified, depending upon the protein, metal ion, and chelating molecules involved.

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