Proc. Natl. Acad. Sci. USA
Vol. 73, No. 11, pp. 3803–3806, November 1976
Chemistry

Covalent attachment of metal chelates to proteins: The stability *in vivo* and *in vitro* of the conjugate of albumin with a chelate of ^{111}indium

(bifunctional chelating agents/reporter groups/radiopharmaceuticals)

CLAUDE F. MEARES*, DAVID A. GOODWIN†, CHARLES S-H. LEUNG*, AMAL Y. GIRGIS*, DAVID J. SILVESTER‡, ADRIAN D. NUNN‡, AND PETER J. LAVENDER‡

*Department of Chemistry, University of California, Davis, California 95616; †Stanford University School of Medicine and Department of Nuclear Medicine, Veterans Administration Hospital, Palo Alto, California 94304; and ‡Medical Cyclotron Unit, Hammersmith Hospital, London, England

Communicated by John D. Baldeschwieler, August 13, 1976

ABSTRACT **Human serum albumin has been conjugated to 1-(*p*-benzenediazonium)-(ethylenedinitrilo)tetraacetic acid, a powerful chelating agent, and radioactive ^{111}indium ions have been added specifically to the chelating groups. The product, with a specific radioactivity of about 1 mCi/mg of protein, was employed as a radiotracer in scintillation scanning studies with human volunteers. Results show that 48 hr after injection, practically all of the label remains attached to albumin. This is confirmed by electrophoresis of serum proteins; 7 days after injection, 85% of the radioactivity in the serum is still in the albumin fraction. These observations agree with *in vitro* studies of the labeled albumin in human serum, where loss of the metal ion from the chelating group to the protein transferrin amounts to less than 3% after 1 week and less than 5% after 2 weeks. Measurements of the distribution of label in mice up to 23 days after injection suggest that metabolism of the labeled protein does not lead to binding of indium ions by transferrin. The binding of indium and other metal ions by transferrin has previously posed a major impediment to the use of metal chelates for *in vivo* diagnostic procedures. Demonstration of the kinetic inertness of the chelate in these experiments suggests the use of related chelates as physical probes of biological systems.**

A metal ion may be attached to a biological molecule by means of a "bifunctional" chelating agent capable of forming a covalent bond to the desired molecule. The conjugation of a derivative of EDTA to a large molecule permits the addition of any of a number of metal ions to form a stable chelate. In principle, this can lead to the utilization of the physical properties of various metal ions in a broad range of applications. Of particular importance is the tagging of certain biological molecules with short-lived radionuclides, and the use of these tagged molecules in medical diagnosis (1).

A case in point is the use of chelates containing radioisotopes of indium for tracer studies. The intravenous injection of such compounds into human patients often leads to the binding of a large fraction of the radioactive metal by the serum protein transferrin, with the result that the observed behavior of the radionuclide is characteristic of the transferrin–indium complex (2, 3). The rapid action of apo-transferrin in removing trivalent iron from chelates is also well known (4, 5). Before biomolecules labeled with indium chelates can be considered valid radioindicators *in vivo*, it must be shown that the chelates are thermodynamically stable or kinetically inert to the extent that loss of indium is negligible.

Previously, we have studied the metabolism of proteins conjugated with indium chelates by measuring the biological lifetime of the label in mice and by observing the distribution of radioactivity among serum proteins in rabbits (1, 6). The results of these studies suggested that the protein-chelate conjugates were reasonably stable *in vivo*, but that as much as 50% of the metal ion might eventually become bound to transferrin and be sequestered in the liver and bone marrow. The present studies were undertaken to provide a detailed account of the behavior of carrier-free amounts of the albumin-chelate conjugate, both *in vivo* and under physiological conditions *in vitro*.

Abbreviation: NO_2Ph-EDTA(In), chelate of indium with 1-(*p*-nitrophenyl)-(ethylenedinitrilo)tetraacetic acid.

MATERIALS AND METHODS

All reagents were the purest commercially available products, used without further purification except where noted. Chromatographic columns and materials were obtained from Bio-Rad Laboratories, Richmond, Calif. 94804.

Azoprotein. The "bifunctional" chelating agent 1-(*p*-benzenediazonium)-(ethylenedinitrilo)tetraacetic acid was prepared as described previously (6). Azoalbumin was prepared by direct addition, at 4°, of a 10-fold molar excess of a 4.4 mM solution of this chelating agent in 0.8 M HCl to an unbuffered solution of human serum albumin (0.1 mM), with the pH kept between 7 and 10 by addition of 1 M NaOH. For the experiments described here, the average albumin molecule contained 1.3 chelating groups, of which approximately 20% were attached to histidine residues and the remainder to lysine residues (practically no azotyrosine was detected). Approximately 40% of the protein-bound EDTA groups were available for the binding of indium ions. These experiments will be described in detail elsewhere (Leung and Meares, submitted for publication).

Chelation of Indium. Because of the affinity between serum albumin and various metal ions (7), the addition of indium ions only to the EDTA groups on azoalbumin requires that the intrinsic metal-binding sites of the protein be masked in some way. It was previously reported (6) that this could be achieved by use of a buffer with metal-chelating properties, 0.1 M sodium citrate at pH 6. Further experiments with albumin which was rendered heavy-metal free by extensive dialysis showed that the native protein could bind carrier-free ^{111}indium ions in 0.1 M sodium citrate at pH 6, but that significant binding did not occur in 0.05 M sodium citrate at pH 3.2. Specific binding of all the indium ions to the EDTA groups on azoalbumin was achieved by the following procedure: 50 μl of a 0.3 mM solution of the azoprotein in 0.1 M sodium citrate at pH 6.5 is cooled in ice, and an equal volume of 0.2 M HCl containing approximately 1 mCi of $^{111}InCl_3$ is added. If necessary, the pH is adjusted to 3.2, and the solution is incubated in an ice-bath for 10 min. The solution is then neutralized with 1 M NaOH, and the radioactive protein is ready for use.

Control experiments show that less than 2% of the indium binds to native albumin under these conditions and that more

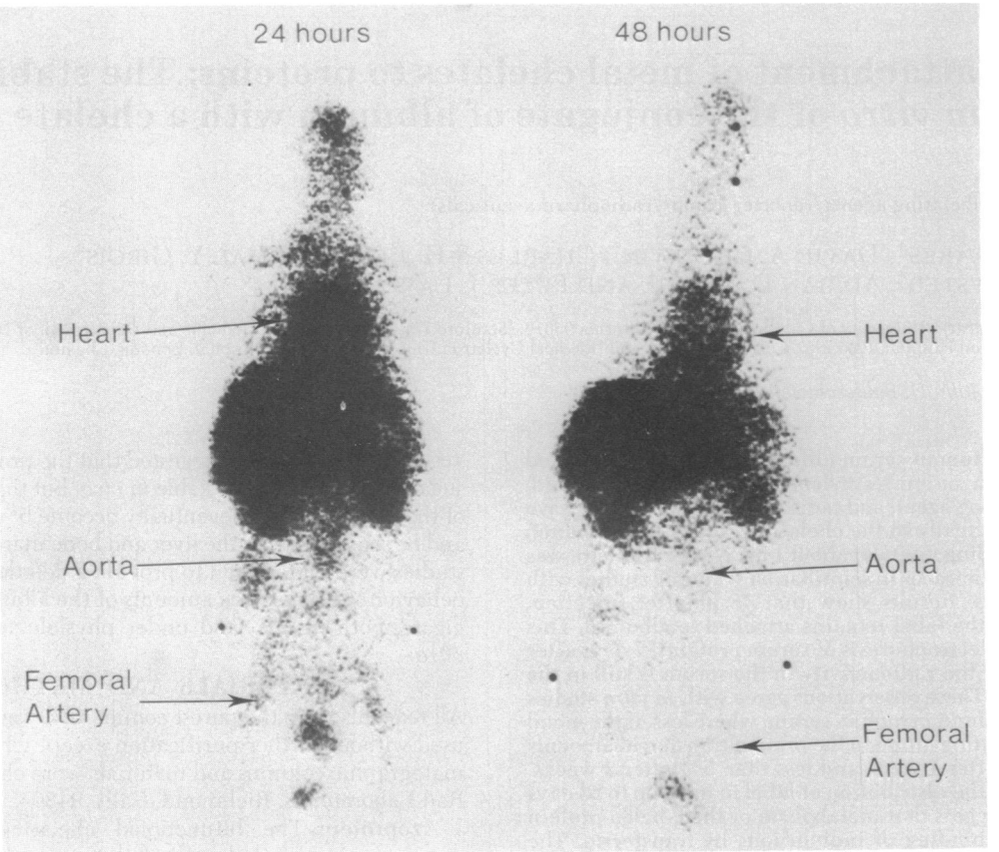

FIG. 1. Anterior whole-body scans of a human subject taken 24 and 48 hr after intravenous injection of 1.4 mCi of ^{111}In-azoalbumin.

than 98% of the indium is bound to the azoalbumin 10 min after mixing. Azoalbumin labeled according to this procedure was used for the studies with human patients. For the *in vitro* experiments, the radioactive azoprotein was passed through a 0.7 × 16 cm column containing Bio-Gel P-300 to remove the small amount of albumin dimer present.

The chelate of indium with 1-(*p*-nitrophenyl)-(ethylenedinitrilo)tetraacetic acid [NO_2Ph-EDTA(^{111}In)] was prepared by adding 5 μl of a 0.1 mM solution of this chelating agent to 200 μl of 0.1 M sodium citrate containing 0.25 mCi of $^{111}InCl_3$.

Human Subjects. Whole body scintillation scanning was carried out 24 and 48 hr after intravenous injection of ^{111}In-labeled azoalbumin, using an Ohio Nuclear Dual 5-inch rectilinear scanner. Two scans are shown in Fig. 1. Daily blood samples were obtained for 2 weeks, and cellulose acetate electrophoresis was carried out on the plasma using barbital buffer at pH 8.6, and applying 18 V/cm for 1 hr. The cellulose acetate strips were cut into 1-cm sections, and each section was assayed for radioactivity in a well scintillation counter. In this system transferrin runs 2 cm from the origin, and albumin between 5 and 6 cm which allows easy separation of these two proteins. Plasma samples were also counted daily in the well counter. Radioactivity remaining in the plasma is shown in Fig. 2; the straight line was determined by a least-squares fit to the last six points, which yielded a half-life of 7.0 ± 0.1 days for the final phase.

Human Serum *In Vitro*. Blood was taken from a healthy adult male donor. It was allowed to clot for 45 min at room temperature and then centrifuged. The serum was drawn off with a pipet and recentrifuged. A mixture of 6 ml of serum and 250 μl of radioactive azoprotein (0.02 mg of azoprotein per ml of serum) was passed through a sterile 0.22 μm filter and apportioned into three sterile vials. A mixture of 6 ml of serum and 100 μl of NO_2Ph-EDTA(^{111}In) was prepared similarly. The vials were placed in an incubator kept at 37 ± 0.5°. An air/CO_2 mixture (95%/5%) was passed through the incubator so that the pH of the serum samples remained at 7.6 ± 0.1 during the experiment.

At intervals, aliquots were taken and analyzed by polyacrylamide gel electrophoresis. The system employed was similar to that of Davis (8), except that the 6-cm running gel contained 6% acrylamide, and no large-pore gel was used. The electrophoresis was done in a Bio-Rad jacketed cell at 2° at 20 V/cm until the bromphenol blue tracking dye was 1 cm from the bottom. For each sample, gels were run in triplicate; one gel was stained with Coomassie brilliant blue, and the other two were frozen, cut into 2-mm slices, and assayed for radioactivity on a Beckman gamma-310 radiation counter.

At the end of 2 weeks, bovine-blood agar petri plates were inoculated with each serum mixture; no bacterial growth was observed after 72 hr at 37°. Addition of $^{111}InCl_3$ to the 2-week-old incubation mixture demonstrated that the transferrin still retained its metal-binding avidity.

Distribution in Mice. To study the time course of the distribution of radioactive azoalbumin *in vivo*, we injected BALB/c mice with approximately 1 μCi of radioactive azoprotein in 0.2 ml of saline. The rate of excretion was determined from whole-body radioactivity measurements in a group of three mice over 23 days, and compared with similar studies using $^{111}InCl_3$ and ^{131}I-albumin. Also, groups of three mice were sacrificed on day 1, 3, 7, 10, and 23 after injection, and the radioactivity in the blood, lungs, liver, spleen, kidneys, muscle, and bone (including marrow) was measured.

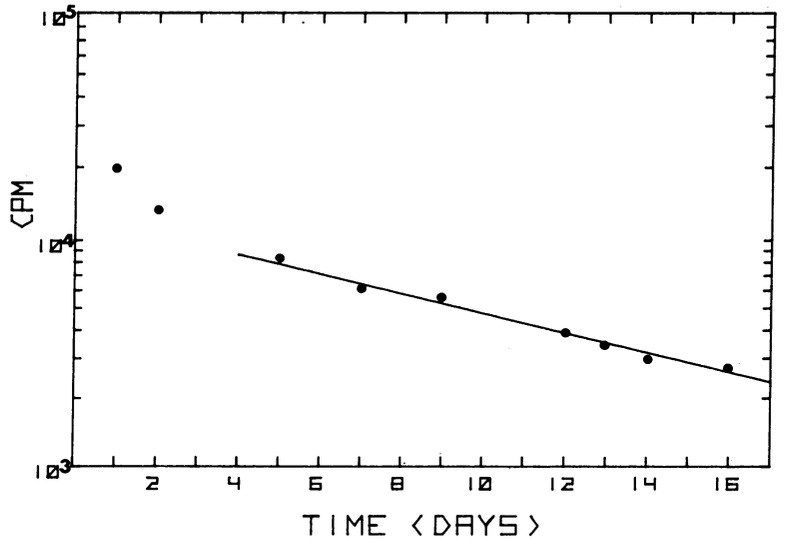

FIG. 2. Semilogarithmic plot showing the disappearance of label from the plasma of a human subject after injection of ^{111}In-azoalbumin. At 7 days, 85% of the radioactivity was still in the albumin fraction. All points have been decay-corrected.

RESULTS

Distribution in man

The plasma disappearance of 1.4 mCi of ^{111}In-labeled human serum albumin was followed for 16 days in a human volunteer who was also studied by scintillation scanning. As shown in Fig. 1, an excellent scan of the whole body showed the large vascular structures to be well outlined over a 48 hr period, with no evidence of the bone marrow accumulation which is seen when ^{111}In-transferrin is formed. The biological half-life in plasma was 7.0 ± 0.1 days (Fig. 2), with 85% of the plasma radioactivity still in the albumin fraction on day 7.

Human serum

After polyacrylamide gel electrophoresis of the serum samples, the stained gels were similar in appearance to those of Davis (8). The bands of the stained gels were studied for changes during the 2-week incubation. The only significant change observed came after 258 hr, when slow evaporation led to some aggregation of the proteins. Measurements of radioactivity versus migration distance obtained from electrophoretic analysis of the serum–NO_2Ph-EDTA(^{111}In) mixture permitted easy determination of the fraction of radioactivity associated with transferrin. As shown in Fig. 3, this quantity is 5% of the total ^{111}In after 333 hr of incubation. It was also observed that a small amount of the intact chelate NO_2Ph-EDTA(^{111}In) migrates with albumin during the electrophoresis.

Electrophoresis of the mixture of radioactive azoalbumin with serum revealed slow separation of some of the indium chelate from albumin, amounting to 6% of the total after 333 hr. Similar behavior has been reported for other azoproteins (9). The time course of this decomposition is shown in Fig. 3; note that the initial mixture contained 2.3% "free" chelate.

The loss of indium ions from azoalbumin to transferrin is difficult to quantitate with this electrophoretic system because the dimer of albumin migrates only slightly faster than trans-

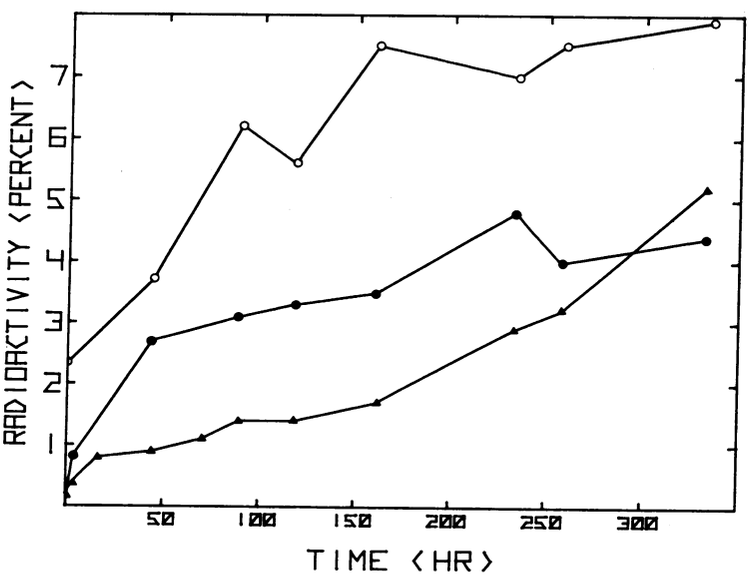

FIG. 3. Time course of the decomposition of NO_2Ph-EDTA(^{111}In) and ^{111}In-azoalbumin in serum. Ordinate gives the percent of the total ^{111}In appearing in each product: ^{111}In-transferrin from NO_2Ph-EDTA(^{111}In) (▲); albumin dimer plus ^{111}In-transferrin from ^{111}In-azoalbumin (●); "free" chelate from ^{111}In-azoalbumin (○).

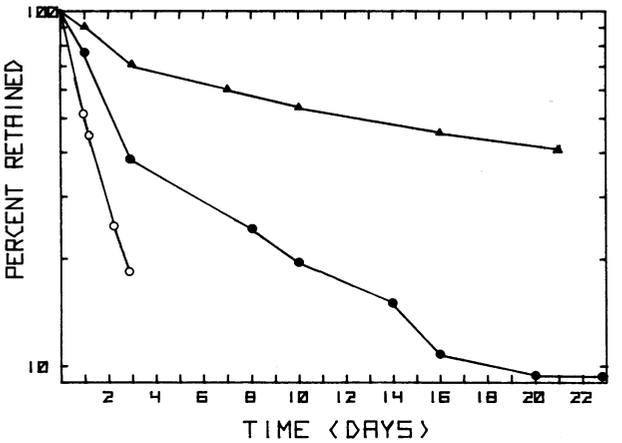

FIG. 4. Semilogarithmic plot showing the amount of radioactivity remaining in BALB/c mice after injection of $^{111}InCl_3$ (▲), ^{111}In-azoalbumin (●), or ^{131}I-albumin (○). All points have been decay-corrected.

ferrin. The *sum* of the radioactivity in the albumin-dimer band and the transferrin band is plotted in Fig. 3. After 2 weeks of incubation, this quantity represents less than 5% of the total ^{111}In, and a significant portion of this appears to be due to the formation of albumin dimers containing ^{111}In-azoalbumin.

Distribution in mice

The retention of radioactive azoalbumin by BALB/c mice is shown in Fig. 4, along with the results of similar measurements for $^{111}InCl_3$ and ^{131}I-albumin. For ^{111}In-azoalbumin, the whole-body retention of radioactivity falls rapidly to about 40% after 3 days and finally reaches a plateau with about 10% of the label retained after 16 days. Radioactivity from ^{111}In-azoalbumin is retained mainly in the liver, with a comparatively small quantity in the marrow; this will be discussed in detail elsewhere. Because of the improved labeling procedure, these results differ from those published previously (1). These experiments suggest that metabolism of the azoalbumin in mice leads mainly to the excretion of intact chelates rather than to the production of indium ions.

DISCUSSION

Upon injection of simple salts or weak chelates of indium into the human circulatory system, the metal rapidly becomes bound to transferrin and then is deposited slowly in the bone marrow (2, 3); trivalent iron is bound and transported in a similar way. The exchange of iron between chelates and transferrin has been studied in some detail, and it has been reported that, under physiological conditions, the loss of iron from many of the most stable chelates to transferrin proceeds rapidly (4, 5). Since iron and indium form chelates of comparable stability with EDTA and related polyaminocarboxylate chelating agents (10), it was expected that exchange of indium from the chelates used in this work to transferrin might also occur at a significant rate.

We have demonstrated by these experiments that, for the bifunctional chelating agents employed, exchange of indium to transferrin proceeds very slowly *in vivo* and *in vitro*. These results provide forceful evidence that the technique of labeling biological molecules with chelates containing radioactive indium has significant applications to nuclear medicine (11, 12).

Chelating agents which can be attached to macromolecules, and which form metal chelates that are stable under physiological conditions, provide a new approach to the study of biological systems. It is expected that $^{+}N_2$Ph-EDTA and related compounds will form stable chelates with metal ions having a variety of spectroscopic and radioactive properties. Such chelates may find many applications as physical probes, or "reporter groups," for investigation of the structural and dynamic properties of biological systems.

We thank Mr. Paul R. Beninger, Mrs. Carol I. Diamanti, and Mr. Jerrold T. Bushberg for assistance. This research was supported by Public Health Service Research Grant no. CA-16861 from the National Cancer Institute and by Veterans Administration Grant no. 3204.

1. Goodwin, D. A., Sundberg, M. W., Diamanti, C. I. & Meares, C. F. (1975) "^{111}Indium-labeled radiopharmaceuticals and their clinical use," in *Radiopharmaceuticals*, eds. Subramanian, G., Rhodes, B. A., Cooper, J. F. & Sodd, V. J. (Society of Nuclear Medicine, Inc., New York), pp. 80–101.
2. Hosain, F., McIntyre, P. A., Poulose, K., Stern, H. S. & Wagner, H. N. (1969) "Binding of trace amounts of ionic indium-113m to plasma transferrin," *Clin. Chim. Acta* **24**, 69–75.
3. Goodwin, D. A., Meares, C. F. & Song, C. H. (1972) "The study of ^{111}In-labeled compounds in mice, using perturbed angular correlations of gamma radiations," *Radiology* **105**, 699–702.
4. Harmuth-Hoene, A. E., Vladar, M. & Ohrtmann, R. (1970) "Iron(III)-exchange between transferrin and chelates *in vitro*," *Chem. Biol. Interact.* **1**, 271–283.
5. Bates, G. W., Billups, C. & Saltman, P. (1967) "The kinetics and mechanism of iron(III) exchange between chelates and transferrin," *J. Biol. Chem.* **242**, 2816–2821.
6. Sundberg, M. W., Meares, C. F., Goodwin, D. A. & Diamanti, C. I. (1974) "Selective binding of metal ions to macromolecules using bifunctional analogs of EDTA," *J. Med. Chem.* **17**, 1304–1307.
7. Breslow, E. (1973) in *Inorganic Biochemistry*, ed. Eichorn, G. L. (Elsevier, New York), pp. 227–249.
8. Davis, B. J. (1964) "Disc electrophoresis-II. Method and application to human serum proteins," *Ann. N.Y. Acad. Sci.* **121**, 404–427.
9. Gelewitz, E. W., Riedeman, W. L. & Klotz, I. M. (1954) "Some quantitative aspects of the reaction of diazonium compounds with serum albumin," *Arch. Biochem. Biophys.* **53**, 411–424.
10. Sillen, L. G. & Martell, A. E., eds. (1964) *Stability Constants of Metal Ion Complexes* (Chemical Society, London).
11. Sundberg, M. W., Meares, C. F., Goodwin, D. A. & Diamanti, C. I. (1974) "Chelating agents for the binding of metal ions to macromolecules," *Nature* **250**, 587–588.
12. Goodwin, D. A., Meares, C. F., Diamanti, C. I. & Sundberg, M. W. (1975) "Bifunctional chelates for radio-pharmaceutical labeling," *Nuclear Medizin* **14**, 365–373.