myoglobin and for one of the Fe(II)'s in deoxyhemerythrin and for a reduced coordination number for the Cu(I)'s in deoxyhemocyanin. For large molecules such as these in which there is limited access of substrate ($O_2$) to the active site, formation rates are fast and probably approach the diffusion-controlled limit, consistent with addition at an unoccupied coordination position. The similar magnitudes of equilibrium constants ($M^{-1}$) in $H_2O$ at 25 °C for myoglobin ($9.7 \times 10^5$), the monomeric form of hemocyanin ($5.7 \times 10^5$), and octameric hemerythrin ($2.5 \times 10^5$) are also noted (Table V).

To summarize, the studies described here add support to the active site structure E, which has been proposed for oxyhemerythrin, with an unusual and at present quite unique form of $O_2$ binding. The kinetic results obtained for myoglobin and hemocyanin in $H_2O$ and $D_2O$ are consistent with known $O_2$-binding properties of these proteins.

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Contribution from the Departments of Medicinal Chemistry, Immunology, and Molecular Pharmacology, Smith Kline and French Laboratories, Philadelphia, Pennsylvania 19081

# Reactions of the Metallodrug Auranofin [(1-Thio-β-D-glucopyranose-2,3,4,6-tetraacetato-*S*)(triethylphosphine)gold] with Biological Ligands Studied by Radioisotope Methodology

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The metallodrug auranofin [(1-thio-β-D-glucopyranose-2,3,4,6-tetraacetato-*S*)(triethylphosphine)gold] is an orally active drug in the treatment of rheumatoid arthritis. The reactivity of auranofin with purified bovine serum albumin (BSA), transferrin, histidine-rich glycoprotein, and metallothionein was studied by using a triple-label radioisotope methodology developed for this purpose. Auranofin was labeled specifically in the triethylphosphine with tritium, in the glucose ring with carbon-14, and with the radionuclide gold-195. The sensitivity of radioisotope detection allowed investigation of ligand-exchange reactions in aqueous solution at physiologically relevant concentrations of drug and biomolecule. By triple-label radioisotope methodology, linked with gel filtration chromatography, it was possible to follow the fate of the gold ion and both ligands in the same experiment. BSA reacted with auranofin to replace the thiosugar ligand and form (triethylphosphine)gold-S-BSA. This complex underwent thiol exchange with cysteine and glutathione but did not react with histidine, methionine, or chloride ion. BSA was coordinated to gold through the thiol of Cys-34 and did not dissociate the phosphine ligand of auranofin. No reaction was observed for auranofin with transferrin or histidine-rich glycoprotein, prototypic metal carrier proteins in the serum that bind essential metals through tyrosine and/or histidine side chains. Metal-free rabbit liver metallothionein I reacted with auranofin to dissociate the thiosugar and phosphine ligands. These studies provide insights into the relationship of structure to efficacy for the metallodrug.

## Introduction

Although metals have been used medicinally for centuries, discovery of the antitumor activity of *cis*-diamminedichloroplatinum(II)[1] and the success of this coordination compound as an anticancer drug[2] has stimulated renewed interest in metal complexes as drugs. The metallodrug auranofin [(1-thio-β-D-glucopyranose-2,3,4,6-tetraacetato-*S*)(triethylphosphine)gold] was recently introduced for the treatment of rheumatoid arthritis. There is little doubt that other metallodrugs will be developed in a variety of therapeutic areas as the field of inorganic medicinal chemistry continues to grow.

It is important to realize that the etiology of rheumatoid arthritis is not well understood and the biological target site(s) that are responsible for the activity of gold metallodrugs are not known. Gold metallodrugs used in the treatment of rheumatoid arthritis are illustrated in Figure 1. These compounds contain gold in the +1 oxidation state. Au(I) is generally coordinated by two ligands in an approximately linear geometry. As suggested by the physical properties of their ligands, Solganal (with thioglucose ligands) and Myochrysine (with thiomalate ligands) are both extremely water-soluble while auranofin (with tetraacetylthioglucose and triethylphosphine ligands) is sparingly soluble in water. Auranofin is an orally active drug while Solganal (gold thioglucose) and Myochrysine (gold sodium thiomalate) are active only when injected.[4-8]

The biological metabolism of metal-ligand complexes is not like the enzymatic modification, degradation, and excretion of organic drugs. Metal-ligand complexes may exchange some or all of their original ligands for biological ligands on contact with biological fluids. In the study of inorganic drug action, knowledge of ligand-exchange reactions in the biological system and the structures of metal-biomolecular complexes can provide a basis for hypotheses about the mechanisms of biological activity. The reactivity of metal complexes also provides critical insights into structure-activity relationships (SAR) to suggest a logical chemical basis for the synthesis of more effective compounds.

Studies of metal-biomolecule complexes usually rely on spectroscopy as a primary research tool.[3] However, spectroscopic methods can be limited by the availability of sufficient quantities of the *biological* material. Millimolar solution concentrations (milligram quantities of biological molecules) are usually required. The detection limits of spectroscopic techniques can also make studies in aqueous solution difficult for lipophilic compounds like auranofin.

In this report we describe the development of a new triple-label radioisotope technique that makes it possible for the first time to follow the ligands and the gold ion of auranofin in the same experiment. We use the technique to investigate the reactivity of auranofin with the purified proteins bovine serum albumin, transferrin, histidine-rich glycoprotein, and metallothionein in

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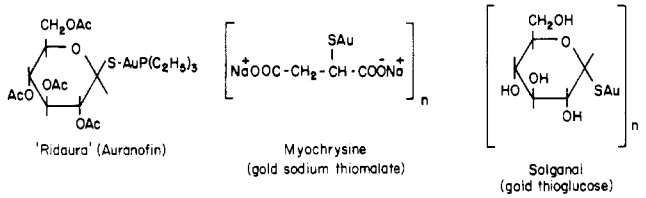


**Figure 1.** Chemical structures of the gold metallodrugs used in the treatemnt of rheumatoid arthritis.

aqueous solution at physiologically relevant concentrations.

## Materials and Methods

Bovine serum albumin (BSA, Pentex fraction V.) and sulfhydryl-modified albumin were obtained from Miles. These proteins contained 0.6 ± 0.05 and 0.002 titratable sulfhydryl groups per mole, determined as previously described.[27] Rabbit liver metallothionein (MTI) was obtained from Sigma as a zinc- and cadmium-containing protein. Transferrin, apotransferrin, amino acids, and other biochemicals used in this study were also obtained from Sigma. Histidine-rich glycoprotein was donated by Dr. Mary Birch.

[$^{195}$Au]Auranofin was synthesized by New England Nuclear. Auranofin, individually labeled in the triethylphosphine with tritium and in the glucose ring with carbon-14, was synthesized by the Department of Radiochemistry at Smith Kline and French Laboratories. The singly labeled compounds were mixed in the appropriate ratio to obtain triply labeled auranofin. The specific activities of each label are given in the legend to Figure 3. Detailed conditions for each of the biological reactions of auranofin are given in the figure legends. Preparation of the liposomes is described in the legend to Figure 5.

Products of reactions of auranofin with biological molecules were separated by gel filtration chromtography, and each fraction was analyzed as described with both a NaI (Tl) crystal $\gamma$ counter (Beckman 7800) and a liquid scintillation counter equipped with a multichannel analyzer (Beckman 9800).

Decay of the radionuclide $^{195}$Au occurred by electron capture and resulted in the emission of platinum K X-rays at 68 keV and $\gamma$ rays at 99 and 130 keV,[9] all of which were detected in a NaI (Tl) crystal $\gamma$ counter with an overall efficiency of about 76% (Figure 2). The absolute disintegration rate per minute (dpm) of $^{195}$Au in the triply labeled sample was determined in a $\gamma$ counter with no interference from the weak $\beta^-$ particles from carbon-14 or tritium by the standard equation

$$\mathrm{dpm} = \frac{\mathrm{cpm} - \mathrm{Bgr}}{E} \tag{1}$$

where $E$ is the fractional counting efficiency for $^{195}$Au.

The decay of $^{195}$Au also resulted in the emission of Auger electrons and internal conversion electrons in the energy range 13–99 keV.[9] These electrons were detected in the liquid scintillation counter with an overall efficiency for $^{195}$Au (in a wide open window) of 94%. The liquid scintillation pulse height spectrum of $^{195}$Au observed in a multichannel analyzer showed extensive overlap with $\beta^-$ scintillation spectra of carbon-14 and tritium (Figure 2) precluding the determination of carbon-14 and tritium by standard double-label methodology in samples that contained all three isotopes. However, because the absolute dpm of $^{195}$Au in a given sample could be measured independently in the $\gamma$ counter, the contribution of counts from $^{195}$Au were subtracted from the summation spectrum of all three isotopes in the liquid scintillation counter by using the equations

$$N_1 = E_{11}\mathrm{dpm}_1 + E_{21}\mathrm{dpm}_2 + E_{31}\mathrm{dpm}_3 \tag{2}$$

$$N_2 = E_{12}\mathrm{dpm}_1 + E_{22}\mathrm{dpm}_2 + E_{32}\mathrm{dpm}_3 \tag{3}$$

$E_{ij}$ represents the fractional counting efficiency for the isotope $i$ in counting channel $j$, where isotopes 1, 2, and 3 are tritium, carbon-14, and gold-195, respectively. Counting channels 1 and 2 were set at 0–400 and 400–650 (Figure 2). The net count rates $N_1$ and $N_2$ are defined as the counts per minute (cpm) observed in each counting channel after background subtraction. The net count rate in each channel is the summation of the absolute DPM of each isotope multiplied by the fractional counting efficiency for the isotope in that channel. The fractional efficiencies $E_{ij}$ for each isotope in each counting channel were determined by the construction of six quench curves using the inflection point of the compton edge of a $^{137}$Cs source as an external standard ($H\#$[10]). Because $\mathrm{dpm}_3$

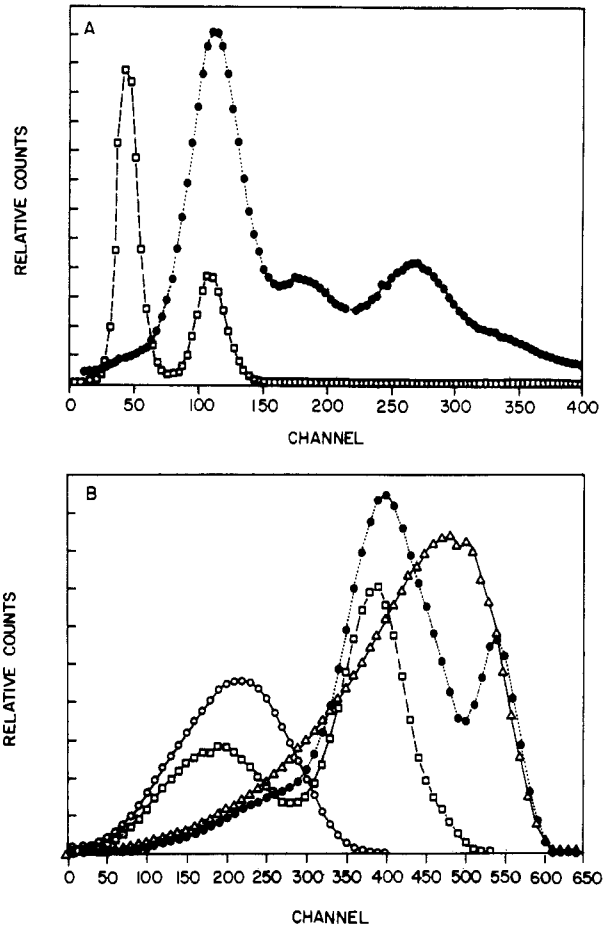


**Figure 2.** (A) Pulse-height spectra for $^{195}$Au (●) and $^{125}$I (□) in a NaI (Tl) crystal $\gamma$ scintillation spectrometer (Beckman 7800). (B) Pulse-height spectra for $^{3}$H (○), $^{14}$C (△), $^{195}$Au (●), and $^{125}$I (□) in a liquid scintillation spectrometer (Beckman 9800). The activity of all four isotopes was 200 000 DPM. The quench parameter ($H\#$), the compton edge of a $^{137}$Cs source,[10] was 80 for all four samples. Isotopes were counted in 10 mL of Aquasol 2 (New England Nuclear) in glass scintillation vials.

was determined independently in the $\gamma$ counter via eq 1, eq 2 and 3 have only two unknowns and can be solved:

$$\mathrm{dpm}_1 = \frac{N_1E_{22} - E_{31}E_{22}\mathrm{dpm}_3 - N_2E_{21} + E_{21}E_{32}\mathrm{dpm}_3}{E_{11}E_{22} - E_{12}E_{21}} \tag{4}$$

$$\mathrm{dpm}_2 = \frac{N_2E_{11} - E_{11}E_{32}\mathrm{dpm}_3 - N_1E_{12} + E_{12}E_{31}\mathrm{dpm}_3}{E_{11}E_{22} - E_{12}E_{21}} \tag{5}$$

The data were handled with a Fortran program that accepted the counting efficiency of the $\gamma$ counter, the background count rate in two channels of the liquid scintillation counter, and the specific activities of all three isotopes. Then, for each HPLC fraction, the counts per minute in the $\gamma$ counter and in each channel of liquid scintillation counter and the quench parameter ($H\#$) for the vial were entered. The program solved eq 1, 4, and 5 using the input data and quench curves stored in the program and then converted the DPM of each isotope to pmol of labeled species in each fraction.

In reactions where it was not possible to measure the concentration of proteins by UV absorbance (such as when the proteins were encapsulated in liposomes), the proteins were labeled with $^{125}$I. In this case triple-label analysis was performed on $^{125}$I, $^{195}$Au, and $^{14}$C. Iodine-125 decayed by electron capture and emitted fluorescent X-rays at 73 keV and a $\gamma$ ray at 35 keV. The $\gamma$-ray peak was sufficiently resolved from the $\gamma$ spectrum of $^{195}$Au for double-label analysis by the equations

$$\mathrm{dpm}_1 = \frac{N_1E_{22} - N_2E_{21}}{E_{22}E_{11} - E_{21}E_{12}} \tag{6}$$

$$\mathrm{dpm}_2 = \frac{N_2E_{11} - N_1E_{12}}{E_{22}E_{11} - E_{21}E_{12}} \tag{7}$$

where $\mathrm{dpm}_1$ = $^{125}$I, $\mathrm{dpm}_2$ = $^{195}$Au, counting channel 1 = 30–80, and

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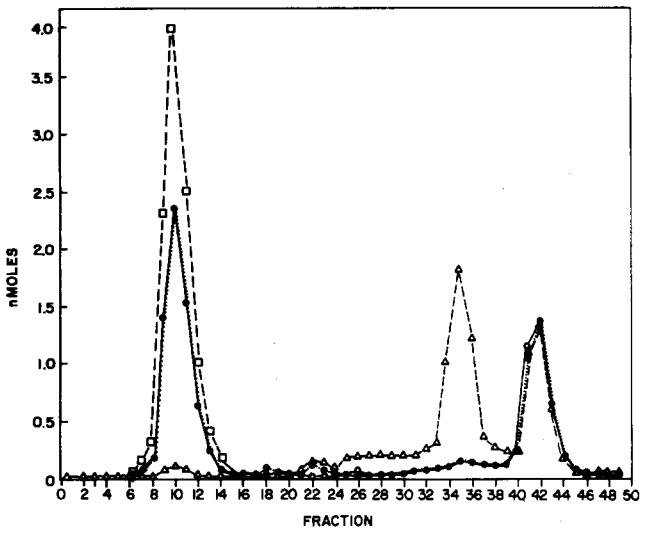


**Figure 3.** Radiochromatograph of the reaction of [$^{14}$C]TATG–S–$^{195}$Au–[$^{3}$H]$PEt_3$ with BSA. The equations derived in the text were used to determine the absolute DPM of each isotope which was converted to nmol of Au (●), $P(Et)_3$ (○), and TATG–SH (△). The protein nmol (□) was determined from $A_{280}$ (at almost every point the symbols for Au and $P(Et)_3$ are superimposed). The specific activities of the triple-labeled auranofin were ($^{195}$Au) $1.4 \times 10^6$ dpm/μmol, ($^{14}$C) $6.9 \times 10^6$ dpm/μmol, and ($^{3}$H) $13.5 \times 10^6$ dpm/μmol. The reaction was carried out as follows: BSA (Pentex fraction V.) at a concentration of 0.15 mM in 0.2 mL of NaHEPES, 100 mM, pH 7.4, was reacted with a stoichiometric amount of auranofin in 10 μL of ethanol. After 20 min at room temperature 90 μL of the reaction mixture was injected on a gel filtration HPLC column (Superose 12, on a Pharmacia FPLC system). After the column void volume (8 mL) 0.5-mL fractions were collected directly into 7-mL glass scintillation vials and counted in the γ counter (Beckman 7800). Next, 5 mL of Aquasol 2 was added and the samples were counted in the liquid scintillation counter (Beckman 9800) as described in the text. Control chromatograms showed that triply labeled auranofin (alone) elutes with the peak at fraction 42, [$^{14}$C]TATG–SH alone elutes with a peak at fraction 35, and BSA alone elutes with a peak at fraction 10.

counting channel 2 = 80–400. Counting efficiencies for each isotope in each counting channel, $E_{ij}$, were determined experimentally. The activity of the remaining β-emitter ($^{14}$C) was determined in the liquid scintillation counter by subtracting the contributions of $^{195}$Au and $^{125}$I from the summation spectrum of all three isotopes as

$$\mathrm{dpm}_3 = \frac{N_1 - E_{21}\mathrm{dpm}_2 - E_{11}\mathrm{dpm}_1}{E_{31}} \qquad (8)$$

## Results

**Serum Proteins.** Albumin plays a physiological role in copper and zinc transport[11] and is present at high concentration in the serum (approximately 0.25 mM). All albumin cysteine residues exist as disulfides with the single exception of Cys-34. The highly purified commercial preparation of BSA used in this study contained approximately 60% titratable reduced Cys-34 SH/1 mol. The remaining 40% of the molecules had Cys-34 blocked as a mixed disulfide. Previous model studies of the binding of Myochrysine[12,13] and of auranofin[14] to bovine serum albumin (BSA) suggest that there is "strong" binding of gold to Cys-34. It is proposed that the gold(I) of Myochrysine retains one thiomalate ligand when bound to Cys-34 and that gold(I) of auranofin retains its triethylphosphine ligand.

The radiochromatogram of the reaction products of stoichiometric auranofin and purified BSA is shown in Figure 3. Sixty percent of the gold and triethylphosphine coeluted with the protein, which was exactly equivalent to the number of reduced sulfhydryl (SH) groups available. The remaining 40% of the gold and triethylphosphine eluted as unreacted auranofin. Less than 2% of the tetraacetylthioglucose (TATG) eluted with the protein. Forty percent was found as unreacted auranofin and 58% as free TATG–SH.

Titration studies showed that no more than 0.6 mol of $AuPEt_3$ bound/1 mol of BSA, even at 20-fold excess auranofin (not shown). When $AuPEt_3$ bound to BSA, stoichiometrically equivalent amounts of TATG–SH were released. There was no evidence for dissociation of triethylphosphine from the gold(I) center. Titration studies with commercially prepared sulfhydryl-modified albumin (in which the free sulfhydryl was blocked) showed that less than 0.02 mol of gold phosphine bound/1 mol of modified protein, even at a 20-fold molar excess of auranofin (not shown).

Our results imply that a thiol-exchange reaction occurs between auranofin and BSA in aqueous solution in which auranofin reacts with the reduced Cys-34 of BSA to form BSA–S–Au–$PEt_3$ and release TATG-SH. Our results suggest that although auranofin reacts with sulfhydryls via a ligand-exchange reaction, it does not react in this manner with other amino acid side chains, such as histidine, tyrosine, aspartic acid, or glutamic acid, which are potential ligands for metals.

This interpretation of results is supported by further studies that showed that auranofin did not react with purified transferrin, apotransferrin or histidine-rich glycoprotein (not shown). No part of the triply labeled sample of auranofin coeluted with the protein peak of transferrin, apotransferrin, or histidine-rich glycoprotein under stoichiometric reaction conditions (like those defined in Figure 3) or with a 20-fold molar excess of auranofin. Apotransferrin has two binding sites for iron(III); each contains two histidine and two tyrosine ligands to bind the metal. Histidine-rich glycoprotein contains about 10% histidine by weight[11,15] and binds zinc and copper.

Albumin may serve as a biological carrier protein for gold and as a source for the biological production of low-molecular-weight gold species. Serum contains low-molecular-weight compounds that are potential ligands for metals, including histidine, methionine, cysteine, glutathione, and chloride ion.[16] To determine whether these ligands could displace gold phosphine from serum albumin or perhaps displace triethylphosphine from the gold center, we used a double-label radioisotope technique to investigate the reactivity of low-molecule-weight ligands with BSA–S–Au–$PEt_3$ labeled with tritium in the triethylphosphine and with the radionuclide $^{195}$Au. Doubly labeled BSA–S–Au–$PEt_3$ was prepared by isolating the desired product from a reaction of excess doubly labeled auranofin with BSA using preparative gel filtration chromatography. Analytical radio HPLC analysis of the doubly labeled biomolecule complex showed no loss of either $^{195}$Au or [$^{3}$H]$PEt_3$ from the protein for at least two weeks after preparation of the complex.

The doubly labeled biomolecule complex (at 0.15 mM concentration) was equilibrated under the reaction conditions defined in Figure 3 with a 10-fold molar excess of histidine, methionine, cysteine, and reduced glutathione and with a 1000-fold (150 mM) excess of NaCl. No reaction was observed with histidine, methionine, or NaCl. However, cysteine and glutathione removed about 50 and 60%, respectively, of the gold phosphine from the biomolecule complex. Stoichiometric quantities of the radiolabels eluted as a low-molecular-weight complex while the biomolecule complex retained a 1:1 tritium to $^{195}$Au stoichiometry following reaction.

These results suggest that cysteine and glutathione removed gold phosphine from a BSA ligand (coordinated to gold through Cys-34) via a ligand-exchange reaction to produce R–S–Au–$PEt_3$. There is no evidence to suggest that these thiols displaced the triethylphosphine ligand. The actual serum concentration of

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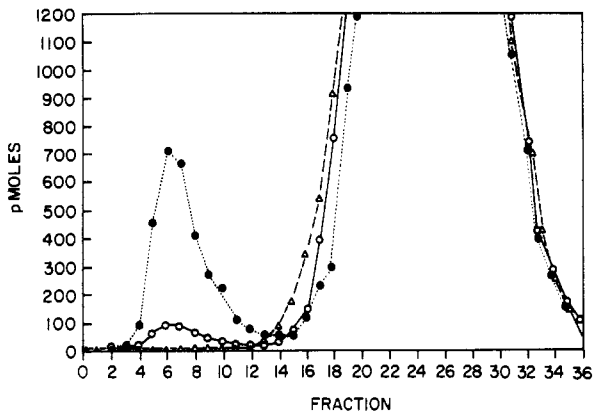


**Figure 4.** Radiochromatograph of the reaction of [$^{14}$C]TATG–S–$^{195}$Au–[$^{3}$H]P(Et)$_3$ with apometallothionein: Au (●), P(Et)$_3$ (○), TATG (△). The specific activities and counting conditions are given in the legend of Figure 3. The reaction was carried out as follows: Apometallothionein was prepared by dissolving metallothionein (rabbit liver MTI, Sigma) in 0.1 N HCl to displace the metals. The apoprotein was separated from the displaced metals by chromatography on Sephadex G25 with 0.1 N HCl as the mobile phase. The protein concentration in the pooled void volume fractions, estimated from the molar extinction coefficient ($\epsilon_{220}$ = 47 300), was 50 μM. To 125 μL of acidified protein solution was added 125 μL of HEPES buffer, 0.5 M, pH 7.4. Triply labeled auranofin was added immediately after the buffer at 20-fold molar excess over protein. After a 1-h incubation at room temperature, 100 μL of the reaction mixture was injected on a Sephadex G25 column with 0.1 M HEPES buffer, pH 7.4, as the mobile phase. Fractions were collected (0.5 mL) and analyzed as in Figure 3.

low-molecular-weight thiols is not high, about 1 and 23 μM for total (both oxidized and reduced) glutathione and cysteine, respectively, while serum albumin is about 250 μM.[11,15,17] Therefore, only small amounts of gold could be coordinated to low-molecular-weight thiols in the serum.

**Metallothionein.** Metallothionein is an intracellular protein that binds metals and is thought to play a major role in zinc and copper metabolism.[18,19] Approximately one-third of the residues of this protein are cysteine. Unlike serum albumin, metallothionein can bind metals as a "multidentate" sulfhydryl ligand.

Rabbit liver metallothionein I is isolated as a mixed cadmium and zinc complex.[18] Auranofin reacted with the metal-free apoprotein prepared from this source (Figure 4) bud did not react with the original cadmium- and zinc-containing protein. The stoichiometry range of reaction mixtures investigated corresponded to a 20–100-fold molar excess of triply labeled auranofin to metallothionein (25 μM). The reduced sulfhydryl content of the apoprotein preparation was not known.

The results of the reaction of auranofin with apoprotein were essentially identical in all studies. The ratio of gold to phosphine associated with the protein fraction was approximately 6:1 (Figure 4). No TATG was associated with the protein. These results showed that the TATG ligand was displaced at all gold centers in the protein complex and that no more than approximately 20% of the gold centers can have retained a triethylphosphine ligand.

**Membrane Permeability to Low-Molecular-Weight Gold Complexes.** We used liposomes prepared from phosphatidylcholine and phosphatidylglycerol as a simple model system[20] to study membrane permeability to low-molecular-weight gold complexes. Large multilamellar liposomes containing BSA (labeled with a tracer amount of [$^{125}$I]BSA) were prepared, separated from unincorporated BSA by gel filtration, and incubated with a 5-fold molar excess of triply labeled auranofin. The liposomes were separated from the incubation solution and analyzed by radioisotope techniques. The molar ratio of gold to BSA in the liposome

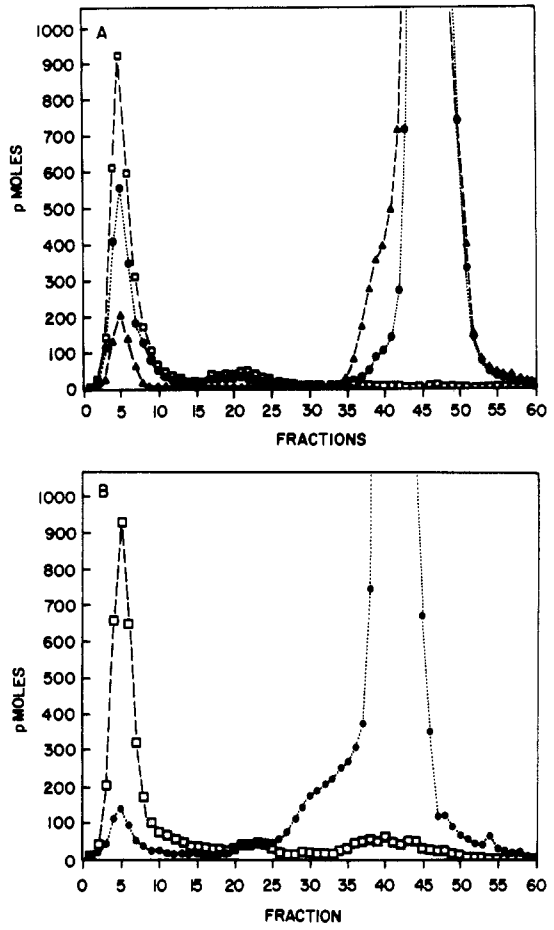


**Figure 5.** Radiochromatograph of the reaction of liposomes containing [$^{125}$I]BSA and (A) [$^{14}$C]TATG–S–$^{195}$Au–P(Et)$_3$ or (B) Cys–S–$^{195}$Au–P(Et)$_3$: $^{125}$I (□), $^{195}$Au (●), $^{14}$C (△). Liposomes were prepared as follows: BSA (100 mg/mL, 1.5 mM) labeled with $^{125}$I by the iodobeads method,[22] specific activity 6 × 10$^6$ dpm/μmol in a 0.1 N HEPES buffer, pH 7.4, was encapsulated in large multilamellar liposomes made of phosphatidylcholine and phosphatidylglycerol (7:3 mole ratio) by the method of ref 20. Liposomes were separated from unincorporated [$^{125}$I]BSA and free lipids on a column (1.6 cm × 17 cm) of Superose 6B Pharmacia) with the same buffer as the mobile phase. Liposomes were pelleted by centrifugation, washed twice with buffer, and resuspended at 1.8 mg [$^{125}$I]BSA/mL, determined by counting an aliquot of the preparation. The liposome entrapment efficiency was 1.9% of the total. Liposomes prepared in this manner eluted as a single peak in the void volume (fraction 5 above) of a Superose 6B column as detected by the UV monitor and γ counting of the fractions. Control chromatograms showed that free [$^{125}$I]BSA eluted with a peak centered at fraction 23 and low-molecular-weight gold chelates eluted from fractions 40–45. To 200 μL of liposomes preparation (5.5 nmol of BSA) was added 27.5 nmol of [$^{14}$C]TATG–S–$^{195}$Au–P(Et)$_3$ or Cys–S–$^{195}$Au–P(Et)$_3$, which is a 5-fold molar excess of metal over BSA. After incubation for 1 h at room temperature, 180 μL of the reaction mixture was injected on the column and 0.5 mL fractions were collected and analyzed. Double-label $^{125}$I and $^{195}$Au analysis was done in the γ counter followed by analysis of $^{14}$C in the liquid scintillation counter. The contributions of $^{195}$Au and $^{125}$I were subtracted from the summation spectrum of all three isotopes by eq 6–8 derived in the text. Cys–S–$^{195}$Au–P(Et)$_3$ was prepared by a ligand-exchange reaction between TATG–S–$^{195}$Au–P(Et)$_3$ and cysteine; the reaction products were separated on a medium-pressure reverse-phase column (HR/PEP, Pharmacia) with 1:1 water/methanol as the mobile phase.

fraction was 0.6 (Figure 5). This result, which was identical with that for the reaction of auranofin and unencapsulated BSA (Figure 3), indicated that auranofin diffused freely across the liposome membrane. However, approximately 37% of the tetraacetylthioglucose ligand, dissociated in a ligand-exchange reaction with Cys-34 of BSA, was also incorporated in the protein/liposome fraction. This observation is consistent with the lipophilic nature of the liposomes and of tetraacetylthioglucose.

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A similar experiment with doubly labeled Cys–S–Au–$PEt_3$, prepared by reaction of a doubly labeled sample of auranofin with excess cysteine, demonstrated that Cys–S–Au–$PEt_3$ was able to penetrate the liposome membrane. The ratio of gold to BSA in the protein fraction of the reaction of Cys–S–Au–$PEt_3$ with unencapsulated BSA was 0.6, which was identical with the reaction of BSA with auranofin. However, in encapsulated BSA the ratio of gold to BSA in the liposome fraction was only 0.16. These results suggest that the liposome is a more formidable barrier for Cys–S–Au–$PEt_3$ than for TATG–S–Au–$PEt_3$. This observation is consistent with the zwitterionic nature of the cysteine ligand.

Several attempts to encapsulate [$^{195}$Au]auranofin in liposomes composed of phosphatidylcholine and phosphatidylglycerol (with and without cholesterol) were unsuccessful. Auranofin diffused freely out of the liposomes during the isolation procedure.

## Conclusions

Knowledge gained in model studies of auranofin in reaction with serum proteins and with the intracellular protein metallothionein is biologically relevant. A substantial fraction (approximately 50%) of the metal–sulfur bonds of the orally active drug auranofin ($10^{-5}$ M) survived exposure to an acidic environment (like the stomach), and the triethylphosphine ligand was not dissociated from the gold(I) center.[21] These observations suggested that a low-molecular-weight species having a gold–triethylphosphine bond can reach the proteins of the serum following oral administration.

Our in vitro studies suggest that auranofin does not interfere with the serum metabolism of essential transition metals at the transport level since it had no apparent affinity for the prototypic metal carrier proteins transferrin and histidine-rich glycoprotein. In vivo studies demonstrated that albumin-bound gold was the major gold-containing serum species following auranofin treatment.[23] This was consistent with the reaction observed for auranofin with BSA in in vitro studies.

Even though the molecular mechanism of gold action in rheumatoid arthritis is unknown, there is evidence to suggest that the macrophage is a therapeutic target cell.[24] In vitro studies presented here showed that auranofin and other low-molecular-weight (triethylphosphine)gold–thiol complexes could diffuse across lipid membranes. Permeability of gold across the macrophage membrane is a prerequisite for activity at cytosolic or nuclear target sites. Protein-bound gold cannot diffuse across lipid membranes. However, small amounts of the gold–phosphine portion of albumin–S–Au–$PEt_3$ may exchange with low-molecular-weight thiols and may subsequently cross the macrophage membrane in a low-molecular-weight form.

A "sulfhydryl shuttle" model has been proposed for the transport of metals into whole cells.[25] In the model, ligand-exchange reactions occur between the exposed sulfhydryls of membranes, proteins, and circulating gold species. Once bound to a membrane sulfhydryl, gold may enter the cytosol by sequential exchange to other sulfhydryl groups on proteins that traverse the membrane. This model is consistent with the results of our in vitro ligand-exchange studies for auranofin.

The in vitro studies presented here also provide insight into the SAR of R–S–Au–$PEt_3$ compounds. A series of thiosugar analogues of auranofin had similar activities in an animal model for rheumatoid arthritis.[26] We suggest that in each case the thiosugar ligand was efficiently displaced by protein sulfhydryls in the same reaction that we observed with auranofin.

The triple-label radioisotope technique used in these studies provides an important new investigative tool for metallodrugs. This tool makes is possible to address questions at an inorganic chemistry level in not only in vitro studies but in vivo studies as well. We foresee its application in a wide range of biological studies.

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**Registry No.** Cys–Au–$PEt_3$, 14243-49-3; auranofin, 34031-32-8; glutathione, 70-18-8.

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