Application of a ^{31}P NMR Chemical Shift: Gold Affinity Correlation to Hemoglobin–Gold Binding and the First Inter-Protein Gold Transfer Reaction

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Abstract: Protein modification, ^{31}P NMR spectroscopy, and stoichiometric measurements on isolated gold–hemoglobin complexes provide definitive evidence for gold binding at the cys-β-93 thiol groups, which are exposed to solvent. Auranofin and two analogues (Et_3PAuX, X = acetylthioglucose, thioglucose, and chloride) react to form $Hb(SAuPEt_3)_n$, $n \leq 2.0$, via exchange of the anionic ligand. The order of reactivity is determined by two factors: the anion's leaving group ability and its affinity for gold(I). Excess Et_3PAuCl also reacts at weaker binding sites (nitrogen or thioether ligands). The oligomeric compound disodium thiomalatogold(I) (AuSTm) reacts with the cys-β-93 thiols more completely than does auranofin and forms a complex with two golds bound. Despite the −3 charge of the CyS–Au–STm grouping, there is no anticooperative effect on the kinetics of binding of the second AuSTm moiety. A correlation of the ^{31}P NMR chemical shifts of thiolato(triethylphosphine)gold(I) complexes in aqueous solution with the affinities of the thiols for gold(I), previously developed for albumin–gold reactions, rationalizes the different reactivities of $Et_3PAuSAtg$ and $Et_3PAuSTg$ with hemoglobin and the competition of $Hb(SH)_2$ and various thiols for gold(I). The first example of facile interprotein gold transfer, from $Hb(SAuPEt_3)_2$ to mercaptalbumin (the SH-containing component of serum albumin), occurs without mediation by small thiols. Its direction was correctly predicted by the NMR shift–gold affinity correlation. The facile nature of this reaction strongly supports two previously developed chemical models of gold biochemistry: (1) equilibration of gold among protein and nonprotein thiols in vivo and (2) the shuttle model for Et_3PAu^+ transport across membranes. The relevance of the ^{31}P NMR correlation and the hemoglobin–gold chemistry for red blood cell accumulation of gold are briefly discussed. The recently reported denaturation of hemoglobin by Et_3PAuCl was found to be important only for concentrations of gold far exceeding that required to saturate the thiol groups, a condition unlikely to be obtained in vivo.

Gold(I) drugs are extensively used in chrysotherapy, the treatment of rheumatoid arthritis, although the metabolism of gold and the mechanism(s) of chrysotherapy are not well understood.[1] Physiological and medicinal carrier ligands significantly affect the distribution of gold(I) in vivo. Gold administered as auranofin [$Et_3PAuSAtg$;[2] 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato-*S*)(triethylphosphine)gold(I)] accumulates inter alia in red blood cells.[3] Gold from oligomeric gold(I) thiolates, such as gold sodium thiomalate (AuSTm; Myochrysine) or gold thioglucose (AuSTg; Solganal), accumulate in the red blood cells (rbc's) of smokers, but not of nonsmokers, because of hydrogen cyanide absorbed from inhaled smoke.[4] The uptake of the triethylphosphinegold(I) moiety of auranofin into red blood cells can be modeled in vitro by reacting Et_3PAuCl with rbc's.[5,6] Gold was found to bind to intracellular glutathione and to a second site assigned as the cys-β-93's of hemoglobin. Thus, it was of interest to compare the binding sites for gold complexes on hemoglobin to the weak and strong gold-binding sites on serum albumin.[7,8]

Recently, we reported a correlation of the ^{31}P NMR shifts for thiolato(triethylphosphine)gold(I) complexes (Et_3PAuSR) with the affinities of the thiols for gold(I).[9] This correlation complements the previously known relationship between the affinities of the thiols (RSH) for gold(I) and their pK_{SH} values.[10] Larger values of δ_P are found for complexes of the thiols with the lowest pK_{SH} values and the greatest affinities for gold(I). Applying this correlation to the δ_P value reported for the hemoglobin complex[5] suggests that it should bind gold less well than serum albumin. Although the in vivo accumulation of Et_3PAu^+ from serum to red blood cells apparently negates the prediction, the in vitro studies which suggested that gold binds to hemoglobin in rbc's used gold concentrations (up to 9 mM) that greatly exceeded the concentrations achieved in therapy (25–50 μM). Therefore, it is important to independently assess the chemical reactivities of various gold(I) compounds toward hemoglobin and compare them with the ability of various drugs to cause gold accumulation in red blood cells.

Because the NMR shift/affinity correlation predicted that the transfer of gold from hemoglobin to albumin should be thermodynamically favorable, we initiated the first examination of interprotein gold transfers in a well-defined medium in order to determine whether the process was kinetically facile.

As this work was being completed, hemoglobin was reported to undergo a novel oxidation to a green colored met–Hb–Au_{20-30} complex upon reaction with large excesses of Et_3PAuCl.[11] This

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(1) (a) Sutton, B. M. *ACS Symp. Ser.* **1983**, *209*, 355–370. (b) Schattenkircher, M.; Muller, W. *Modern Aspects of Gold Therapy*; Karger: Basel, 1983.

(2) Abbreviations: AAS, atomic absorption spectroscopy; AlbSH, mercaptalbumin; Au_i/Hb and Au_b/Hb, the ratios of gold initially added (i) or bound (b) to hemoglobin; AtgSH, 2,3,4,6-tetra-*O*-acetyl-β-1-D-thioglucose; AuSTm, gold sodium thiomalate; AuSTg, gold thioglucose; b-Hb, iodoacetamide modified Hb; cys, cysteine; EDTA, ethylenediaminetetraacetic acid; ErSH, ergothionine; GtSH, glutathione; Hb, hemoglobin, HbCO, carboxyhemoglobin; HbO_2, oxyhemoglobin; MOPS, 3-[*N*-morpholino]propanesulfonic acid; RBC, red blood cells; TgSH, β-1-D-thioglucose.

(3) Herlinger, J. D.; Alsen, C.; Beress, R.; Hecker, U.; Weikert, W. *J. Rheumatol.* **1982**, *9* (Suppl. 8), 81–89.

(4) Lewis, D.; Capell, H. A.; MacNiel, C. J.; Iqbal, M. S.; Brown, D. H.; Smith, W. E. *Ann. Rheum. Dis.* **1983**, *42*, 566–570. (b) Graham, G. G.; Haavisto, T. M.; Jones, H. M.; Champion, G. D. *Biochem. Pharmacol.* **1984**, *33*, 1257–1262.

(5) Malik, N. A.; Otiko, G.; Sadler, P. J. *J. Inorg. Biochem.* **1980**, *12*, 317–322.

(6) Razi, M. T.; Otiko, G.; Sadler, P. J. *ACS Symp. Ser.* **1983**, *209*, 371–384.

(7) Coffer, M. T.; Shaw, C. F., III; Eidsness, M. K.; Watkins, J. W. II; Elder, R. C. *Inorg. Chem.* **1986**, *25*, 333–340.

(8) Shaw, C. F., III; Schaeffer, N. A.; Elder, R. C.; Eidsness, M. K.; Trooster, J. M.; Calis, G. J. M. *J. Am. Chem. Soc.* **1984**, *106*, 3511–3521.

(9) Coffer, M. T.; Shaw, C. F., III; Hormann, A. L.; Mirabelli, C. K.; Crooke, S. T. *J. Inorg. Biochem.* **1987**, *30*, 177–187.

(10) Isab, A. A.; Sadler, P. J. *J Chem. Soc., Dalton Trans.* **1982**, 135–142.

(11) Grootveld, M. C.; Otiko, G.; Sadler, P. J.; Cammack, R. *J. Inorg. Biochem.* **1986**, *27*, 1–5.

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reaction, at stoichiometric and substoichiometric concentrations of Et_3PAuCl, is carefully reexamined here to address the question of gold binding to the cys-β-93 sulfhydryls.

Experimental Section

Materials. HbO_2 was obtained from outdated whole blood or red blood cells by washing isolated rbc's three times with 2 vol of isotonic saline and centrifugation at 40 000*g* for 15 min. Washed cells were lysed with 2 vol of doubly distilled H_2O for 45 min at room temperature and centrifuged at 40 000*g* for 30 min. The supernatant was dialyzed two times against 10 vol of buffer (20 mM MOPS; 150 mM KCl or $LiClO_4$; 5 mM EDTA; pH 7.4) over 48 h. Aliquots of the 1.1–1.3 mM Hb obtained were stored under argon at −4 °C.

HbCO was generated by passing CO over Hb solutions in septum-sealed reaction vessels into which $Na_2S_2O_4$ was syringed. The samples were flushed for an additional 20 min; then the HbCO concentration was determined spectrophotometrically from the extinction coefficient at 419 nm.[12]

Sulfhydryl-modified Hb, b-Hb, was prepared by adding a 100-fold excess of solid ICH_2CONH_2 to 25 mL of 1.0 mM Hb. Following a 15-min incubation at room temperature and centrifugation at 2000*g*, the supernatant was concentrated to 5 mL and chromatographed on a Sephadex G-100 column. The Hb fractions were concentrated on an Amicon ultrafiltration cell to the desired concentration and stored under Ar, as above. Modification of the protein thiols was confirmed spectrophotometrically using the 4,4′-dithiopyridine assay.[13]

Reagents were obtained as follows: Et_3PAuCl and auranofin were generously supplied by Smith Kline and French Laboratories. $Et_3PAuSTg$ (deacetylated auranofin) was prepared as previously described and stored at −4 °C in methanolic solution.[9] From Aldrich Chemical Co. (Milwaukee) were obtained AuSTm, EDTA, 4,4′-dithiopyridine, $OP(OCH_3)_3$, D_2O (99.8%), reduced glutathione, and iodoacetamide; from Sigma Biochemicals (St Louis), Sephadex G-100-120, *l*-ergothioneine and fatty acid free bovine serum albumin; from U.S. Biochemical Corp (Cleveland), MOPS. All other reagents were reagent grade or better.

Chromatography. Methanolic solutions of the Au complexes were added dropwise to the Hb solution (in 20 mM MOPS; 150 mM KCl or $LiClO_4$; 5 mM EDTA; pH 7.4). The content of organic solvent in the final sample did not exceed 15%. The Hb concentrations were 0.82–1.14 mM and Au, 1.8–11.2 mM. Aliquots (1–2 mL) were chromatographed, immediately or after incubation at 4 °C, on a Sephadex G-100 column. The fractions were analyzed spectrophotometrically for Hb (using ϵ_{308} = 63 300 L/mol cm or the appropriate extinction coefficients for the α, β, or γ bands of HbO_2 or HbCO[12,14]). Gold concentrations were determined on an IL-357 atomic absorption spectrometer calibrated with serial dilutions of a Spex gold standard. Protein-containing fractions were isolated, concentrated by ultrafiltration in an Amicon cell, and stored frozen under Ar or N_2 for ^{31}P NMR spectroscopy.

^{31}P NMR Spectroscopy. Broad-band proton-decoupled ^{31}P NMR spectra were obtained immediately after preparation of the samples and were acquired at 101.3 MHz on a Bruker WM 250-MHz spectrometer. Typical acquisition parameters were 45° pulse, 0.54-s repetition time, and 16K data points. The spectral window was −30 to 110 ppm. Chemical shifts were determined and are reported relative to internal $OP(OCH_3)_3$ (δ_P = 2.74 ppm vs 85% H_3PO_4). D_2O in a coaxial insert provided a field-frequency lock. The increased concentrations of Hb utilized for the spectroscopy samples, 2.6–3.7 mM, allowed rapid spectrum acquisition, generally within 1–3 h. For the competition studies, the ErSH or GtSH concentration was exactly half that of the HbO_2.

Spectrophotometric Titration of HbO_2 with Et_3PAuCl. Et_3PAuCl (9.69 mM in methanol) was incrementally added to 250 mL of 15.5 μM HbO_2, yielding solutions of various stoichiometries (0.25–15 Au/Hb tetramer). Aliquots were withdrawn after each addition and reserved for spectrophotometric analysis. The samples were incubated for 30–60 min to assure completeness of reaction, then analyzed spectrophotometrically (250–650 nm) and finally diluted 10-fold to read A_{414}.

AuSTm Binding Kinetics. AuSTm (1.00 mM) and HbO_2 (100 μM) were incubated at 29 °C in MOPS/KCl/EDTA buffer, pH 7.4. Aliquots were withdrawn at 30-s time intervals and at 10 and 30 min, then fractionated over a Sephadex G-50 column (1 × 25 cm). Fractions (2.0 mL) were analyzed for Hb and Au content. The calculated Au_b/Hb ratios were reproducible to ±0.05 Au/Hb. First-order treatment of the data yielded a linear plot through 90% of the reaction.

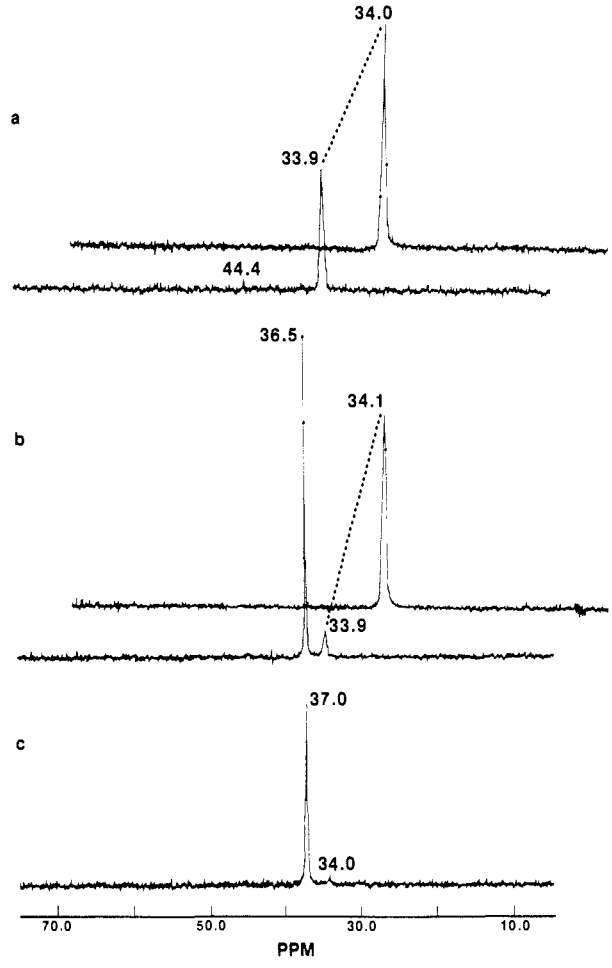


Figure 1. $\{^1H\}$–^{31}P NMR spectra of HbO_2 reactions with (a) Et_3PAuCl, b) $Et_3PAuSTg$, and (c) $Et_3PAuSatg$. Reaction stoichiometries: Au_i/Hb = 2.0; [Hb] = 3.5–3.6 mM in MOPS/KCl/EDTA buffer, pH 7.4. Offset spectra in (a) and (b) are for chromatographically isolated Hb-$(AuPEt_3)_n$. $(MeO)_3PO$ (0.00 ppm) was used as the internal standard.

Results

To determine the influence of the anionic ligands, X, of Et_3PAuX complexes on their reactivity toward hemoglobin, the reactions of $Et_3PAuSAtg$, $Et_3PAuSTg$, and Et_3PAuCl were compared. After mixing 2 equiv of each compound with oxyhemoglobin, HbO_2, the resulting hemoglobin–gold complexes were immediately isolated by gel-exclusion chromatography. The ratios of gold bound to hemoglobin, Au_b/Hb, were 0.30, 0.54, and 1.76, respectively (Table I). If the mixtures were allowed to stand for 24 or 48 h, there was no further reaction. Thus, the order of reactivity of the auranofin analogues toward HbO_2 is $Et_3PAuSAtg < Et_3PAuSTg \ll Et_3PAuCl$. Carboxyhemoglobin, HbCO, was similarly examined (Table I), and the same order of reactivity was observed.

The proton-decoupled ^{31}P NMR spectra (Figure 1) for the stoichiometric Et_3PAuX/HbO_2 reaction mixtures (Au_i/Hb = 2.0) each contained a resonance at 34.0 ppm (versus $OP(OMe)_3$). This signal increased in intensity with the reactivity of the complex. It was the only resonance detected in the Et_3PAuCl-containing sample, while signals for unreacted $Et_3PAuStg$ (36.3 ppm) and $Et_3PAuSAtg$ (37.0 ppm) were observed for the corresponding samples. Spectra obtained after isolating the gold–hemoglobin complexes (Table I; Figure 1 inserts) had only the 34.0-ppm resonance, confirming its assignment as an Hb–$AuPEt_3$ adduct formed via equilibrium displacement of the anionic ligands.

Although the 34.0-ppm chemical shift is low for an Et_3PAuSR complex,[9,15] it was previously suggested by Sadler to represent

(12) Antonini, E.; Brunori, M., unpublished data cited in: Neuberger, A.; Latum, E. L., Eds.; *Hemoglobin and Myoglobin in Their Reactions with Ligands*; North Holland: Amsterdam, 1966; p 19.
(13) Ampulski, R.; Ayers, V.; Morell, S. *Anal. Biochem.* **1969**, *32*, 163–169.
(14) Sugita, Y.; Nagai, M.; Yoneyama, Y. *J. Biol. Chem.* **1971**, *246*, 383–388.

Table I. Gold Binding Ratios and ^{31}P NMR Shifts of Chromatographically Isolated Human Hemoglobin–Gold Complexes Prepared in Vitro[a]

	reactants		hemoglobin–gold complex	
complex	protein	Au_i/Hb	Au_b/Hb[b]	δ_P[c]
$Et_3PAuSAtg$	HbO_2	2.1	0.30	34.0
	HbCO	3.2	0.19	
$Et_3PAuSTg$	HbO_2	2.1	0.54	34.0
	HbO_2	10.7	1.67	34.0
	HbCO	2.1	0.44	
	b-Hb	2.8	0.19	
Et_3PAuCl	HbO_2	1.9	1.76	34.0
	HbO_2	5.0	*d*	
	$HbO_2/LiClO_4$	4.0	3.54/1.83[e]	
	HbCO	2.1	1.92	
	b-Hb	1.4	1.07	
AuSTm	HbO_2	1.0	0.82	
	HbO_2	2.0	0.99	
	HbO_2	8.7	2.03	
	HbCO	1.9	0.50	
	b-Hb	4.0	0.03	

[a] [Hb] ≈ 0.8–1.2 mM in 20 mM MOPS/150 mM KCl/5 mM EDTA, pH 7.4. [b] Gold binding ratios determined by AAS (Au) and UV–visible spectroscopy after removal of free gold over Sephadex G-100. [c] δ_P vs internal $OP(OCH_3)_3$. [d] Extensive precipitation precluded isolation and analysis of the product. [e] $LiClO_4$ was substituted for KCl in the buffer; during concentration of the Hb fractions precipitation of gold and Hb reduced the Au_b/Hb value.

Et_3PAu^+ coordinated at the solvent-accessible (β-93) cysteines of Hb.[5,6] This assignment was tested by modifying the cysteines with iodoacetamide and reacting the modified hemoglobin (b-Hb) with Et_3PAuCl. The Au_b/b-Hb ratio, 1.07 (Table I), determined after isolating the sufhydryl-modified hemoglobin complex, indicates that gold binding was decreased but not completely eliminated by blocking the sufhydryl group. To determine whether Et_3PAu^+ was reacting at nonthiol sites of the b-Hb, ^{31}P NMR spectra were acquired sequentially over a 5-h time period. The 34.0-ppm peak is absent in each case (Figure 2b), supporting its assignment to $Hb(SAuPEt_3)_n$. The broad resonances at 25 and 28 ppm in the Et_3PAuCl reaction mixture (Figure 2b) are attributed to weakly ligated Et_3PAu^+. Et_3PAuCl (31.5 ppm) was not detected. The 28- and 25-ppm chemical shifts are in the region expected for nitrogen or thioether complexes,[9,15] and may represent Et_3PAu^+ coordinated at methionine, histidine, lysine, or possibly the peptide backbone.

At the protein concentrations (1–3 mM) used in obtaining Figure 2b, the Et_3PAuCl caused slow precipitation of the b-Hb. Simultaneously, the 28- and 25-ppm peaks assigned to weakly bound gold (Figure 2b) decreased in intensity over time while the 44.5-ppm resonance due to $Au(PEt_3)_2^+$ increased. Thus, Et_3P may be diplaced from Hb-bound Et_3PAu^+ by other protein ligands with concomitant destabilization and aggregation of the protein.

$Et_3PAuSTg$ is much less reactive than Et_3PAuCl toward b-Hb (Figure 2a; Table I). The Au_b/b-Hb ratio of chromatographically isolated complex was 0.19. The ^{31}P NMR spectrum of the $Et_3PAuSTg$/b-Hb reaction mixture did not contain the 36.5-ppm resonance of unreacted $Et_3PAuSTg$ but rather a broad signal at 35.4 ppm and a small $Au(PEt_3)_2^+$ resonance at 44 ppm. Assuming the 35.4-ppm signal is due to rapid exchange of Et_3PAu^+ between the weak binding sites and thioglucose and using the Au_i/b-Hb ratio given in Table I, one obtains an estimated gold binding ratio of 0.5–0.6. The smaller value determined after gel filtration, 0.19, may result from dissociation of the weakly bound gold during chromatography.

When $LiClO_4$ was substituted for KCl to control ionic strength, the binding of Et_3PAu^+ to HbO_2 increased. The Au_b/Hb ratio was 3.4 using $LiClO_4$, compared to 1.1 using KCl (Table I). Thus, the reaction of Et_3PAuCl at sites other than the β-93 cysteines is reduced by high chloride concentrations, confirming the equilibrium nature of the reactions.

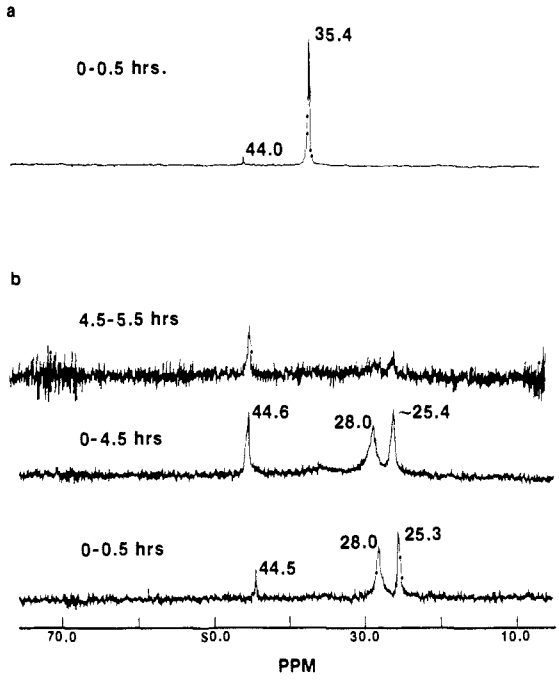


Figure 2. $\{^1H\}$–^{31}P NMR spectra of sulfhydryl-modified hemoglobin (b-Hb, 3.0 mM) after reaction with (a) $Et_3PAuSTg$ (25.5 mM) and (b) Et_3PAuCl (6.4 mM). In the Et_3PAuCl reaction, $(Et_3P)_2Au^+$ increased over 5 h, while the Et_3PAu^+ associated with the weak sites decreased as protein precipitated. The spectrum of the $Et_3PAuSTg$ reaction remained unchanged.

Et_3PAuCl, at mole ratios of 10 to 60 Au/Hb, oxidizes HbO_2, yielding a novel met-Hb complex with numerous gold ions bound: met-Hb-Au_{20-30}.[11] The cys-β-93 residues were described as "not critical" in this reaction, although it was not established whether gold does bind to them. Since Sadler et al.[11] did not report any data points between 0 and 10 Au/Hb (Figure 3, ref 9), we repeated the reaction at 18 data points in the range 0–15 Au/Hb and under the same conditions. As shown in Figure 3, conversion to met-Hb, monitored by the appearance of its 631-nm absorption and by the decreases in the HbO_2 absorbances at 414, 541, and 576 nm, became extensive only after the stoichiometry of added gold significantly exceeded 2:1. Thus, the cys-β-93 tight-binding sites are saturated before gold binding at other sites begins to alter the protein structure and oxidation state.

The competition of hemoglobin and glutathione (GtSH, pK_{SH} = 8.7[16]) or ergothioneine (ErSH, pK_{SH} = 10.8[17]) for gold(I) was examined by ^{31}P NMR. The concentrations of ErSH and GtSH in the RBC are 150–600 μM and ~2.5 mM, respectively, while that of Hb is ~4mM.[18,19] The relationship between affinity of Au(I) for thiols and thiol acidity (pK_{SH})[10] predicts the GtSH should compete more effectively than ErSH for Au(I). The ^{31}P NMR spectra of the samples containing 0.5 or 2.0 equiv of Et_3PAuCl and 0.5 equiv of GtSH per Hb tetramer (Figure 4a) exhibited resonances for $Et_3PAuSGt$ (36.2 ppm) and $Hb(SAuPEt_3)_n$ (34.0 ppm). With 0.5 equiv of gold present, the $Et_3PAuSGt$ concentration greatly exceeded that of $Hb(SAuPEt_3)_n$, even though the Hb thiols were present in fourfold excess over GtSH. Use of 2 equiv of Et_3PAuCl (Figure 4b) saturated the glutathione and reversed the relative concentrations of $Et_3PAuSGt$ (36.2 ppm) and $Hb(SAuPEt_3)_n$ (34.0 ppm).

Upon substituting ErSH for GtSH under identical conditions, only the $Hb(SAuPEt_3)_n$ signal was apparent (34.0 ppm, Figure

(15) Kinsch, E. M.; Stephan, D. W. *Inorg. Chim. Acta* **1984**, *91*, 263–267.
(16) Sillen, L. G.; Martell, A. E. *Stability Constants*, Suppl. No. 1, The Chemical Society: London, 1971; p 655.
(17) Stanounik, B.; Tisler, M. *Anal. Biochem.* **1964**, *9*, 68–74.
(18) Melville, D. B. In Harris, R. S.; Marpian, G. F.; Thimann, K. V., Eds.; *Vitamins and Hormones*; Academic Press: New York, 1959; Vol. 17, pp 155–204.
(19) Beutler, E. *Red Cell Metabolism: A Manual of Biochemical Methods* Grune and Stratton: New York, 1975; p 113.

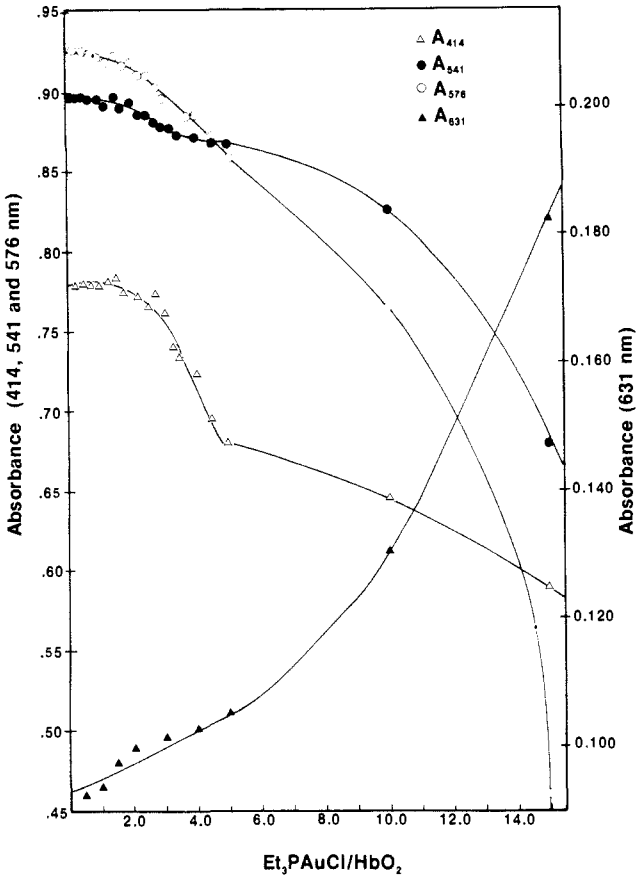


Figure 3. Spectrophotometric titration of HbO_2 (15.5 μM) with Et_3PAuCl. The decrease in the HbO_2 α, β, and Soret bands (576, 541, and 414 nm, respectively) and the appearance of the met-Hb α band (631 nm) occur primarily after the stoichiometry exceeds 2:1.

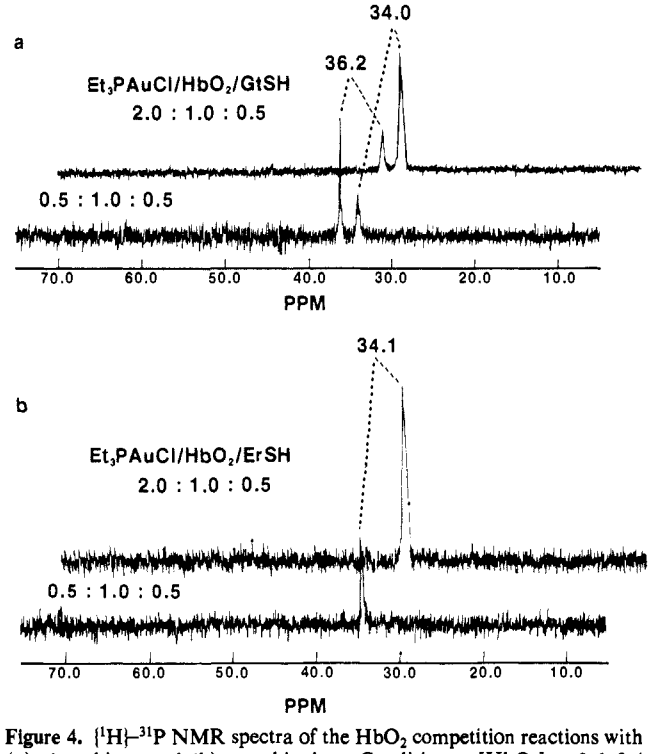


Figure 4. $\{^1H\}$-^{31}P NMR spectra of the HbO_2 competition reactions with (a) glutathione and (b) ergothionine. Conditions: $[HbO_2]$ = 2.6–3.4 mM; RSH/HbO_2 = 0.500; Et_3PAuCl/HbO_2 = 0.50 and 2.00. Note the dramatic change in the relative intensities of $Hb(SAuPEt_3)_n$ and the thermodynamically favored $GtSAuPEt_3$ when GtSH becomes saturated. No signal was detected for $ErSAuPEt_3$ (34.6 ppm), indicating that ErSH has a low affinity for gold(I).

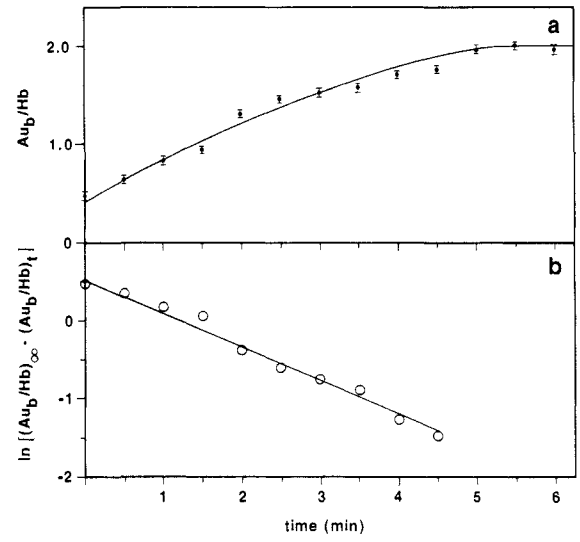


Figure 5. Kinetics of AuSTm binding to HbO_2: 1 mM AuSTm and 100 μM HbO_2 were incubated at 29 °C in 20 mM MOPS/150mM KCl/5mM KCl, pH 7.4. At 30-s intervals up to 6 min and after 10 and 30 min, aliquots were fractionated by gel-exclusion chromatography. The fractions were analyzed for Au and Hb content: (a) Au_b/Hb vs time; (b) ln $[(Au_b/Hb)_\infty - (Au_b/Hb)_t]$ vs time.

4b). The absence of the $Et_3PAuSEr$ resonance, independently determined to be 34.6 ppm, even after adding 2 equiv of Et_3PAuCl per Hb, demonstrates that ErSH cannot effectively compete with Hb for Au(I).

The competition experiments effectively test the correlation of the ^{31}P NMR chemical shifts for the Et_3PAuSR complexes with the affinities of the thiols for gold(I) and their pK_{SH} values.[9] The results indicate that the correlation is valid for aliphatic thiols (e.g, GtSH) but not for ErSH. The latter anomaly is attributed to the thiol–thione equilibrium of ErSH (see Discussion).

The correlation predicts that serum albumin (δ_p 38.8 ppm for $AlbSAuPEt_3$) should have a greater affinity than hemoglobin (δ_p 34.0 ppm for $Hb(SAuPEt_3)_n$) for gold(I). To test this prediction and determine whether interprotein transfer of Et_3PAu^+ can occur without a mediating agent such as GtSH, $Hb(SAuPEt_3)_{1.7}$ and sufficient bovine serum albumin to contain an equivalent of mercaptalbumin (AlbSH/Au = 1.0/1.0) were mixed. The sample was monitored by ^{31}P NMR. Before the albumin was added, only the 34.0-ppm resonance of the $Hb(AuPEt_3)_n$ adduct was present. One hour after AlbSH addition, only $AlbSAuPEt_3$ (38.8 ppm) was detected. Thus, complete transfer of Et_3PAu^+ from hemoglobin to albumin occurred rapidly and without mediation by nonprotein thiols.

The reaction of AuSTm, an oligomeric gold(I) thiolate, with HbO_2 is an equilibrium process. The Au_b/Hb ratio saturates at a value of 2.0, even with excess gold present (Table I, Figure 5). The decrease in Au_b/Hb to 0.03 as a consequence of iodoacetamide modification of the β-93 cysteines (Table I) establishes them as the only AuSTm binding sites. This result and the maximum Au_b/Hb value of 2.0 demonstrate that AuSTm does not react at the additional sites populated by Et_3PAuCl and $Et_3PAuSTg$. AuSTm, unlike the phosphine complexes, is considerably less reactive toward HbCO than toward HbO_2 (Table I).

AuSTm and HbO_2 react at rates measurable by conventional methods. Chromatographic separation is the most convenient technique since the Hb and gold UV–visible absorbances are relatively insensitive to the reaction occurring. Figure 5a shows data for the reaction of 1 mM AuSTm with 100 μM of HbO_2. The Au_b/Hb ratio saturates at 2.0 ± 0.05 after approximately 5 min. If the net −3 charge of the first β93-CyS-Au-STm unit were to inhibit the formation of the second, the reaction kinetics should display anticooperativity. To test this possibility, the data were plotted as ln $[(Au_b/Hb)_\infty - (Au_b/Hb)_t]$ vs t (Figure 5b). The single slope for the time interval required for Au_b/Hb to reach 2 eliminates the possibility that the second gold binds at a significantly slower rate. The slope corresponds to a pseudo-first-

order rate constant of 8.9×10^{-3} s^{-1}.

Discussion

The reactivity of the Et_3PAuX complexes toward HbO_2, HbCO and b-Hb is $Et_3PAuCl \gg Et_3PAuSTg > Et_3PAuSAtg$. This order can be attributed to the affinities of the anionic ligands for gold(I) [AtgSH > TgSH > Cl^-] and their leaving group abilities [Cl^- > TgSH > AtgSH]. The ^{31}P NMR results confirm that each complex reacts at the β-93 thiols via exchange of the anionic ligand to yield the identical product, $Hb(SAuPEt_3)_n$ (34.0 ppm, Figure 1):

$$n\mathrm{Et_3PAuX} + \mathrm{Hb(SH)_2} \rightleftarrows \mathrm{Hb(SAuPEt_3)}_n + n\mathrm{X^-} + n\mathrm{H^+}$$

The Et_3PAuCl reaction is nearly stoichiometric, consistent with general strengths of Au–Cl and Au–SR binding. The same relative reactivity was observed in reactions of RAW 264.7 macrophage cells[20] and serum albumin[9] with these compounds.

The insignificant binding of gold(I) from $Et_3PAuSAtg$ or $Et_3PAuSTg$ contrasts with the analogous reactions at the cys-34 thiol of serum albumin where all three complexes react stoichiometrically to yield $AlbSAuPEt_3$. The differences between the Hb and albumin reactions demonstrate a substantial influence of protein environment and tertiary structure on sulfhydryl group reactivity toward gold(I) complexes. A priori, one might expect the solvent-accessible β-93 thiols of Hb to be more reactive toward Au(I) than is cys-34 of albumin which is buried in a 10-Å cleft. The fact that the converse is true suggests that the albumin crevice and the local environment of cys-34 greatly enhance its affinity for gold(I). The anomalously low pK_{SH} value of cys-34, estimated to be less than 5,[21] may result from the same factors.

Hemoglobin binds only one AuSTm unit per β-93 thiol, unlike albumin which can bind up to six AuSTm units per cys-34 thiol.[8] Each thiomalate ligand carries a −2 charge due to its carboxylate groups. The failure of Hb to bind additional AuSTm moieties may result from the same factors which render the cys-β-93 residues less reactive toward negative reagents (e.g., iodoacetate and DTNB) than toward neutral or positive analogues (e.g., iodoacetamide and pyridine disulfide).[22]

The reaction of $Hb(SAuPEt_3)_n$ with albumin is the first demonstration that gold can be readily transferred between proteins. In this case it is a facile process and occurs without mediation or catalysis by small thiols such as glutathione. The reaction proceeds essentially to completion, since no hemoglobin-bound phosphine was detected in the ^{31}P NMR spectrum:

$$\mathrm{Hb(SAuPEt_3)}_n + n\mathrm{AlbSH} \rightarrow \mathrm{Hb(SH)_2} + n\mathrm{AlbSAuPEt_3}$$

The facility of this reaction provides strong support for two proposed models of gold biochemistry: (1) the equilibration of gold(I) among proteins and nonprotein sulfhydryl groups[23,24] and (2) the "sulfhydryl shuttling" model proposed for cellular membrane transport of gold compounds.[25] The equilibration model was developed to explain the similarity between the gold distributions obtained by in vivo administration of gold(I) and by in vitro addition of gold(I) to isolated tissues or fluids.[23,24] The sulfhydryl shuttle model proposes that Et_3PAu^+ is transported across cell membranes by two interprotein transfers. First, it is transferred from protein sulfhydryl groups in the external media to membrane surface sulfhydryl groups that translocate it into the cell interior. There it is transferred to the sulfhydryl groups of cytoplasmic proteins within the cell.[25]

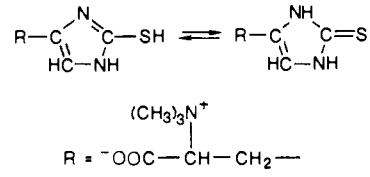
Three of the results obtained in this study provide striking confirmation of the recently reported correlation between ^{31}P NMR chemical shifts for Et_3PAuSR complexes, affinity of thiols for Au(I) and pK_{SH} values[9] (Table II). They are (1) the direction of the hemoglobin to albumin gold transfer, (2) the relative affinities of albumin and hemoglobin for auranofin and deacetylated auranofin, and (3) the hemoglobin–glutathione competition for Et_3PAuCl. Thus, auranofin (δ_p 37.0 ppm), deacetylated auranofin (δ_p 36.3 ppm), and $Hb(SAuPEt_3)_n$ (δ_p 34.0 ppm) react completely with mercaptalbumin to form $AlbSAuPEt_3$ (δ_p 38.8 ppm).[7] In contrast, the reactions of auranofin and deacetylated auranofin with hemoglobin are incomplete, as predicted by their δ_p values.

Table II. Correlation of ^{31}P NMR Chemical Shifts with pK_{SH} Values

Et_3PAuSR	δ_p[a]	ref	pK_{SH}	ref
$Hb(SAuPEt_3)_x$	34.0	*b*		
$ErSAuPEt_3$	34.6	*b*	10.8	17
$GtSAuPEt_3$	35.8	9	8.9	16
$TgSAuPEt_3$	36.3	9	7.6	27
$AtgSAuPEt_3$	37.0	7	6.4	28
$AlbSAuPEt_3$	38.8	7	<5	21

[a] In aqueous soln vs $OP(OCH_3)_3$. [b] This work.

The preferential binding to Hb in the $Hb(SH)_2$/ErSH competition, Figure 4b, was not correctly predicted by the correlation between ^{31}P NMR shifts and affinity for gold(I). This discrepancy may be attributed to the tautomerism of the 2-thiol histidine moiety which binds gold less avidly than aliphatic thiols:



Thus, the correlation is applicable to aliphatic thiols, but should not be extended to 2-thiol histidines or aromatic thiols where thioketo–thiol tautomerism provides a competing equilibrium.

The reaction of Et_3PAuCl and deacetylated auranofin at the weak binding sites of iodoacetamide-modified Hb is consistent with the denaturation study of Sadler et al.[11] The transformation to met-Hb, which they detected after binding of many gold equivalents per heme,[11] occurs to a negligible extent until the cys-β-93 cysteines are saturated with gold. Neither met-Hb formation nor the binding at the weak, nonthiol sites (δ_p 25, 28 ppm) would be expected under the conditions prevailing during chrysotherapy.

The results of this study have important implications for the in vivo biochemistry of gold in the blood. The affinity of Hb for AuSTm is far greater than for auranofin (Table I), which is exactly the opposite of the propensity of the drugs or their metabolites to accumulte in rbc's in vivo or in vitro.[3] Thus, rbc accumulation of gold from different drugs is not determined by the thermodynamic affinities of the drugs for Hb.

As suggested by the ^{31}P NMR chemical shift of $GtSAuPEt_3$, GtSH is a more favorable binding site than Hb. The GtSH, $Hb(SH)_2$, and ErSH concentrations in rbc's are ~2.5, 4, and 0.15–0.60, mM, respectively. Thus, high concentrations of Et_3PAuCl will saturate the glutathione forming $GtSAuPEt_3$ (2.5 mM), then bind to Hb forming $Hb(SAuPEt_3)_n$ as shown in Figure 4 and previously.[6] When the gold concentration is limiting (e.g., during chrysotherapy, where serum gold is 15–50 μM), Et_3PAu^+ will bind to GtSH in preference to $Hb(SH)_2$ or ErSH (Figure 4). GtSH does not, however, compete effectively with serum albumin for gold(I).[9] Furthermore, Et_3PAu^+ transport into red cells occurs by a passive shuttling mechanism rather than by active transport,[25] and must occur against a gradient established by the unusual thermodynamic stability of serum albumin gold complexes. Thus, there must be high-affinity binding sites, other than

(20) Snyder, R. M.; Mirabelli, C. K.; Crooke, S. T. *Biochem. Pharmacol.* **1987**, *36*, 647–654.
(21) Lewis, S. D.; Misra, D. C.; Shafer, J. A. *Biochemistry* **1980**, *19*, 267–273.
(22) Garel, C.; Beuzard, Y.; Thillet, J.; Domenget, C.; Martin, J.; Galacteros, F.; Rosa, J. *J. Biochem.* **1982**, *123*, 513–519.
(23) Thompson, H. O.; Blaszak, J.; Knudtson, C. J.; Shaw, C. F., III. *Bioinorg. Chem.* **1978**, *9*, 375–388.
(24) Shaw, C. F., III. *Inorg. Perspect. Biol. Med.* **1979**, *2*, 278–355.
(25) Snyder, R. M.; Mirabelli, C. K.; Crooke, S. T. *Biochem. Pharmacol.* **1986**, *35*, 923–932.
(26) Shaw, C. F., III; Schmitz, G.; Thompson, H. O.; Witkiewicz, P. J. *Inorg. Biochem.* **1979**, *10*, 317–330.
(27) Isab, A. A. Ph.D. Thesis, University of London, 1978.
(28) Hall, W. W. Smith Kline and French Laboratories, unpublished results.

hemoglobin or glutathione and as yet unidentified, which account for the uptake of serum gold into the rbc's.

Summary

Definitive evidence has been obtained for the binding of gold complexes at cys-β-93 of hemoglobin. AuSTm, $Et_3PAuSAtg$, and $Et_3PAuSTg$ bind only at cys-β-93, while Et_3PAuCl binds there and also at additional weak binding sites. The correlation between thiol pK_{SH}, ^{31}P NMR chemical shift, and affinity of Au(I) for various thiols successfully rationalizes the extent of the reactions of auranofin and its analogues with proteins. The NMR correlation predicted the direction of the interprotein (hemoglobin to albumin) gold transfer reaction. Because gold(I) ligand exchange reactions are facile, this correlation should prove useful in elucidating the mechanism of action of gold(I) drugs and their distributions in vivo. Predicting of the position of equilibria using ^{31}P NMR chemical shifts will be especially helpful when pK_{SH} values are not available. The comparison between the high affinity of albumin and the low affinity of hemoglobin for gold(I) and the greater affinity of hemoglobin for AuSTm than for auranofin establish that the accumulation of gold metabolites of auranofin in red blood cells is not driven by thermodynamic factors related to hemoglobin-gold complex formation.

Registry No. Et_3PAuCl, 15529-90-5; Et_3PAuST_g, 34031-29-3; AuSTm, 112070-13-0; Au, 7440-57-5; Cys, 52-90-4; auranofin, 34031-32-8.

The Nigericin-Mediated Transport of Sodium and Potassium Ions through Phospholipid Bilayers Studied by ^{23}Na and ^{39}K NMR Spectroscopy

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Abstract: Addition of nigericin to preparations of large unilamellar vesicles made from egg yolk phosphatidylcholine in sodium or potassium chloride solution gives rise to dynamic ^{23}Na and ^{39}K NMR spectra. The dynamic spectra arise from the nigericin-mediated transport of the metal ions through the membrane. The kinetics of the transport are followed as a function of metal ion and nigericin concentrations and are compatible with a model in which one nigericin molecule transports one metal ion. The data allow the extraction of the rate constants for the association and dissociation of the nigericin/metal complex in the water/membrane interface and the evaluation of the stability constants for complex formation in the interface. The rate-determining step in the transport process is dissociation of the complex. Although sodium on its own is transported more rapidly, the greater stability of the potassium complex means that potassium is preferentially transported when both metal ions are present.

The transport of materials through the limiting membranes of living cells is a process of fundamental importance that has attracted widespread attention.[1] Essential materials such as amino acids, nucleotides, sugars, and ions are transported by membrane-bound transport systems. Quantitatively the major metal ions in such transport processes are Na^+ and K^+. Many enzyme systems that transport organic substrates also require cotransport of these metal ions. Maintenance of the transmembrane gradients of Na^+ and K^+ is vital. This is ensured by the Na^+/K^+ pumping enzyme, NaKATPase.[2]

An alternative mode of transport of metal ions exists when ionophoric materials are present.[3-7] Many ionophores, notably monensin, nigericin, and valinomycin, are active as antibiotics[8] and also have marked physiological effects in mammals. These properties presumably result from the dissipation of transmembrane ion gradients. It is believed that these materials transport ions through membranes in the form of their complex with the metal.[9] The speeding up of the transport they induce cannot be compensated by the normal mechanisms available to the cell.

Ionophoric materials can be divided into two categories, anionic and neutral. The anionic ionophores have an ionizable group, generally a carboxylic acid, and can thus form a metal/ionophore complex which is electrically neutral. The molecular structures of these ionophores are characterized by a chain of tetrahydrofuran and tetrahydropyran rings terminated at one end by a carboxylic acid group and at the other by one or more hydroxyl groups which hydrogen bond round to the acid end in the metal ion complex. Examples include monensin and nigericin (1). Neutral ionophores have no ionizable group and thus form a charged complex with the alkali metal ion. Examples include valinomycin, enniatin, the crown ethers, and the cryptands. The rate at which neutral ionophores transport ions through membranes is potentially limited by charge buildup in the membrane or cotransport of anions. These limitations do not apply to anionic ionophores where the transporting species is neutral.

The detailed model we employ for the transport of a metal ion (M^+) through a membrane is based on the classical mobile carrier system of Willbrandt and Rosenberg[10] as presented by Painter and Pressman.[9] For an anionic ionophore the transport through a phospholipid bilayer, e.g., phosphatidylcholine (PC), can be broken down into three distinct phases, each of which may in its turn be more complex. These steps are illustrated in Figure 1, and the validity of this model for transport is investigated in this

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(1) Wright, J. K.; Seckler, R.; Overath, P. *Annu. Rev. Biochem.* **1986**, *55*, 225-248.
(2) Glynn, I. M.; Karlish, S. J. D. *Annu. Rev. Physiol.* **1975**, *37*, 13-55.
(3) See, for example, articles in *Top. Curr. Chem.*, Vols. 98, 101, and 121.
(4) Lehn, J. M. *Struct. Bonding (Berlin)* **1973**, *16*, 1-69.
(5) Simon, W.; Morf, W. E.; Meier, P. C. *Struct. Bonding (Berlin)* **1973**, *16*, 113-160.
(6) Pressman, B. C. *Annu. Rev. Biochem.* **1976**, *45*, 501-530.
(7) Ovchinnikov, Y. A. *Eur. J. Biochem.* **1979**, *94*, 321-336.
(8) See, for example, articles in: *Metal Ions in Biological Systems*; Sigel, H., Ed.; Marcel Decker: New York, 1985; Vol. 19.
(9) For a review see: Painter, G. R.; Pressman, B. C. *Top. Curr. Chem.* **1982**, *101*, 83-110.
(10) Wilbrandt, W.; Rosenberg, T. *Pharmacol. Rev.* **1961**, *13*, 109-183.

0002-7863/88/1510-0734$01.50/0