hexane eluent and recrystallization from pentane, 520 mg (25%) of **6u** as colorless crystals. HRMS (EI, 70 eV) for $C_{20}H_{20}O_2$ (M^+): calcd 292.146 320, found 292.145 777 5.

1,1,1-Trichlorooct-3-yn-2-one (13). At −78 °C, **9d** [from 1-hexyne (1 mL, 8.7 mmol) and 2.5 M *n*-BuLi (2.8 mL, 7 mmol) in THF (20 mL) at −78 °C] was added to a mechanically stirred suspension of **8h** (4.07 g, 7.7 mmol) and *t*-BuNO (32 mg, 0.4 mmol) in THF (100 mL). The reaction mixture was allowed to warm up to room temperature, and the solvent was removed in vacuo. The white solid residue was treated with ligroin, lithium trichloroacetate was filtered off, and the filtrate was concentrated in vacuo. Column chromatography on silica gel (50 g) with hexane gave iodobenzene (1.17 g, 82%) as the first fraction and 280 mg of a not completely pure second fraction. Chromatography on silica gel (50 g) of the second fraction, with hexane, yielded 200 mg (12%) of **13** as a colorless oil. ^{1}H NMR ($CDCl_3$, TMS): δ 0.93–0.98 (m, 3 H), 1.42–1.55 (m, 2 H), 1.60–1.70 (m, 2 H), 1.45–1.50 (m, 2 H). ^{13}C NMR ($CDCl_3$): δ 13.38, 19.12, 21.88, 29.20 (*n*-Bu), 74.33 (C≡C), 95.40 (CCl_3), 104.24 (C≡C), 168.28 (C=O). IR (neat): 2210 (vs. C≡C), 1705 (vs, C=O), 1195 (vs, C—O), 900, 805, 765, 730, 660 cm^{-1}. MS (CI, CH_4 as ionizing gas): 229 (15, M^+ + H + 2), 227 (15, M^+ + H), 193 (4, 229 − Cl), 191 (6, 227 − Cl), 165 (2, 193 − CO), 163 (3, 191 − CO), 129 (2, 165 − Cl), 127 (5, 163 − Cl), 109 (100, C_7H_9O). HRMS (CI, CH_4 as ionizing gas) for $C_8H_{10}OCl_3$ (M^+ + H): calcd 226.979 860, found 226.978 386.

Reversibly and Irreversibly Formed Products from the Reactions of Mercaptalbumin (AlbSH) with Et_3PAuCN and of $AlbSAuPEt_3$ with HCN

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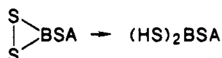
Abstract: The reactions of $AlbSAuPEt_3$ (cysteinyl-34-(triethylphosphine)gold(I) albumin) with HCN and of mercaptalbumin (AlbSH) with Et_3PAuCN were examined by ^{13}C and ^{31}P NMR spectroscopy, gel exclusion chromatography, and $^{14}CN^-$ radiotracer methods. The reaction of HCN with $AlbSAuPEt_3$ yields irreversibly formed products, Et_3PO, $[Au(CN)_2]^-$, and a protein–gold complex, AlbSAuX, where X ≠ CN^- or PEt_3. During the reaction, a transient, reversibly formed product, Et_3PAuCN, is generated but reacts further to yield the final products. The reaction of independently prepared Et_3PAuCN with AlbSH gives the same set of irreversibly formed products. Here, also, transient, reversibly formed products, $AlbSAuPEt_3$ and HCN, are observed. Thus, each set of reactants yields the same irreversibly formed products and the other set as transient, equilibrium products. These results are explained by proposing a common three-coordinate transition state, $[AlbSAu(PEt_3)CN^-]^{\ddagger}$, for the equilibration of the two sets of reactants/products and the formation of the irreversibly formed products. Et_3P and $AlbSAuCN^-$ are proposed to be intermediates leading from the transition state to the irreversibly formed products. We report the first direct evidence that the oxidation of Et_3P to Et_3PO is accompanied by the reduction of the albumin disulfide bonds



$[(Et_3P)_2Au]^+$, which is generated in solutions of Et_3PAuCN via ligand disproportionation, reacts with AlbSH to produce $AlbSAuPEt_3$ and Et_3PO. The new thiol groups, $(HS)_2BSA$, generated by the phosphine oxidation, react with the $[(Et_3P)_2Au]^+$ to produce $(Et_3PAuS)BSA$, which is characterized by a ^{31}P NMR resonance at 35.8 ppm. The relevance of these biochemical reactions to the gold metabolism of cigarette smoking chrysotherapy patients is discussed.

The metabolism of anti-arthritic gold drugs can be altered by the absorption of HCN from tobacco smoke.[2,3] Gold metabolites of the oligomeric drugs such as aurothiomalate (AuSTm[4]) enter the red blood cells of smoking patients but not of nonsmoking patients[2,3] or laboratory animals.[5,6] Smokers have earlier and more frequent toxic reactions to gold drugs.[2] Metabolites of auranofin ((triethylphosphine)(2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato-*S*)gold(I), $Et_3PAuSATg$) enter rbc's irrespective of smoking habits.[3,7] To our knowledge, ligand exchange reactions of auranofin metabolites and HCN, which might induce other, more subtle metabolic changes, have not been investigated.

Cyanide binds to gold very tightly (log β_2 = 36.6),[8] although exchange of free HCN (the form present at neutral pH due to hydrolysis of CN^-) with bound cyanide is very rapid. Independent studies by this laboratory and Sadler's demonstrate that the equilibrium competition of HCN and thiols for gold(I) favors cyanide and that the mixed ligand complexes, $[RSAuCN^-]$, form

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(4) Abbreviations: AAS = atomic absorption spectroscopy; $AlbSAuPEt_3$ = cysteinyl-34-(triethylphosphine)gold(I) albumin; AlbSAuSTm = cysteinyl-34-(thiomalato)gold(I) albumin; AlbSH = mercaptalbumin; ATgSH = 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-thioglucose; AuSTm = sodium ((*S*)-thiomalato)aurate(I); BSA = microheterogenous bovine serum albumin; DTNB = 5,5′-dithiobis(2-nitrobenzoic acid); $Et_3PAuSATg$ or AF = Auranofin (triethylphosphine)(2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato-*S*)gold(I); GtHS = reduced glutathione; HSA = human serum albumin; RSH = reduced thiol; TMP = trimethyl phosphate; TSP = 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid, sodium salt.

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and disproportionate to $[(RS)_2Au]^-$ and $[Au(CN)_2]^-$.[9,10] Trialkyl- and triarylcyano(phosphine)gold(I) complexes also disproportionate in solution:[11]

$$2\,Et_3PAuCN \rightleftharpoons [(Et_3P)_2Au]^+ + [Au(CN)_2]^- \quad (1)$$

In the blood serum, gold is bound principally to albumin.[5] In vitro studies demonstrate that AuSTm and Et$_3$PAuSATg react with the mercaptalbumin component, AlbSH, of serum albumin to form AlbSAuSTm[4] and AlbSAuPEt$_3$,[4] respectively.[12,13] The gold(I) is bound to a cysteine residue (Cys-34)[12-14] located in a crevice estimated to be 10-Å deep.[15] The albumin can, therefore, be considered a thiolate ligand. Recently, gold-bridged dimers of albumin have been proposed to explain thermodynamic data.[16] Although slowly formed mercury-bridged analogues[17] have been reported, previous attempts to isolate the gold dimers by gel chromatography yielded only negative results.[12,13] Thus, ligand scrambling reactions analogous to reaction 1 seem unlikely for AlbSAuSTm or AlbSAuPEt$_3$.

With few exceptions, the reactions of various albumin–gold species are not well known. Et$_3$PO, the metabolite of the phosphine moiety of auranofin, forms over 24 to 72 h in various species.[7] This process can be modelled in vitro by the reactions of thiols with AlbSAuPEt$_3$.[14] The unusually great affinity of the Cys-34 thiol for gold(I) apparently labilizes the phosphine facilitating its displacement and subsequent oxidation.[14] The thiols (RSH) with the greatest affinity for gold promote the reaction most effectively.[14] The disulfide bonds of the albumin have been proposed to act as the oxidants.[14,18,19]

$$AlbSAuPEt_3 + RSH \rightleftharpoons AlbSAuSR^- + H^+ + Et_3P \xrightarrow{[O]} Et_3PO \quad (2)$$

On the basis of this background, the reactions of HCN with AlbSAuPEt$_3$ were examined to (1) study the competition of HCN with the phosphine and protein thiolate ligands of AlbSAuPEt$_3$, (2) test the prediction that cyanides would generate Et$_3$PO from AlbSAuPEt$_3$ more effectively than thiols, and (3) develop a biochemical model for the metabolism of auranofin in smoking chrysotherapy patients. In this work, as previously,[12-14] we have used bovine serum albumin (BSA) because it has more reproducible chemical and physical properties (especially the mercaptalbumin content, i.e., thiol titer) than human serum albumin (HSA). The amino acid sequences of HSA and BSA are strongly homologous, indicating that similar chemistry will be observed. In particular, the free thiol, Cys-34, has an invariant position.[20]

Experimental Section

Materials. Sephadexes G-50 and G-100 and 5,5'-dithiobis(2-nitrobenzoic acid) were obtained from Sigma Biochemicals, BSA (fatty acid free Lot No. 10282520-49) from Boehringer Mannheim Biochemicals Co., and D$_2$O (99.8% *d*) and CH$_3$OD (99.5% d) from Aldrich Chemicals, and K^{13}CN (99% enriched) was purchased from Cambridge Isotope Laboratories. Na^{14}CN (8 mCi/mmol) from ICN Chemical and Radioisotope Division was diluted with ordinary KCN to form a stock solution (10.3 mM; 0.976 mCi/mmol). Et$_3$PAuCl and [(Et$_3$P)$_2$Au]Cl were generous gifts from SKF Laboratories. Et$_3$PAu^{13}CN (25% enriched) was synthesized as previously described.[11]

AlbSAuPEt$_3$. BSA of known SH titer was reacted with Et$_3$PAuSATg as previously described.[12] The resulting AlbSAuPEt$_3$ was chromatographically isolated, analyzed for gold content, and stored frozen, as a lyophilized solid, until used. Typical preparations contained 0.5–0.7 mol fraction of AlbSAuPEt$_3$ compared to total BSA. ^{31}P NMR analysis established that Et$_3$PAu$^+$ was bound only at the free sulfhydryl group (Cys-34) and not at the weaker, nitrogen binding sites.[12] The stoichiometries of subsequent reactions are based on the gold content.

^{13}C and ^{31}P NMR Measurements. ^{13}C and ^{31}P NMR spectra were obtained in the desired deuteriated solvents on a Bruker WM 250 multinuclear NMR spectrometer at 62.9 and 101.3 MHz, respectively. The pulse angles used were 45° or less and the temperature was 295 K. ^{31}P NMR chemical shifts were measured relative to trimethyl phosphate (TMP) as an internal reference. ^{13}C NMR chemical shifts were measured relative to the sodium salt of 3-(trimethylsilyl)propionic-*2,2,3,3-d$_4$* (TSP) as an external reference. For the ^{13}C NMR spectra, 15 000 to 40 000 scans were accumulated except in the experiments involving detection of the transient, equilibrium products where approximately 40 000 scans were accrued.

Analyses. Gold was quantitated by flame atomic absorption spectroscopy (AAS), albumin by its UV absorption at 278 nm (ϵ_{278} = 39 600 L·mol^{-1}·cm^{-1}), and the albumin SH titer with DTNB,[21] as previously described.[12] Scintillation counting (^{14}C) was performed on a Beckman LS 3801 Liquid Scintillation System. Cocktails were prepared by vigorously mixing 100-μL aliquots of the aqueous analyte solutions with 1.0 mL of H$_2$O and 3.0 mL of Optifluor cocktail mixture (Packard Instruments).

^{13}C and ^{31}P NMR Titrations of AlbSAuPEt$_3$ with H^{13}CN. Aliquots (40, 20, and 30 μL) of K^{13}CN solution (10.0 mM in D$_2$O) were added sequentially to 2.0 mL of AlbSAuPEt$_3$ (0.69 Au/BSA; 2.68 mM BSA in 100 mM NH$_4$HCO$_3$ buffer, pH 7.9). ^{13}C NMR spectral acquisition began immediately after each addition.

AlbSAuPEt$_3$ (0.69 Au/BSA; 1.47 mM BSA in 1.5 mL of D$_2$O/100 mM NH$_4$HCO$_3$) was similarly titrated with K^{13}CN and followed by ^{31}P NMR. Acquisitions were begun immediately after the addition of K^{13}CN and completed within 30 min.

Chromatographic Titration of AlbSAuPEt$_3$ with KCN. Aliquots (0.50 mL) of AlbSAuPEt$_3$ (934 μM BSA; 0.69 Au/BSA) were treated with various amounts of 10.0 mM KCN, then applied to a Sephadex G-50 column (1 × 25 cm), and eluted with 100 mM NH$_4$HCO$_3$ buffer, pH 7.9, at 15–25 mL/h. Fractions were collected and analyzed for their gold and albumin contents, from which the ratio of gold remaining bound to the BSA was determined.

Similar titrations with AlbSAuPEt$_3$ (Au/BSA = 0.69; 75.7 μM BSA) and K^{14}CN (10.3 mM; 0.976 mCi/mmol) were carried out as above, analyzing for gold, albumin, and [^{14}C]cyanide content of the fractions.

Detection of Transient Products. Method 1: To AlbSAuPEt$_3$ (Au/BSA = 0.59; 1.69 mM BSA in 100 mM NH$_4$HCO$_3$ buffer) was added equimolar KCN. The solution was immediately applied to a 1.5 × 40 cm Sephadex G-100 column at 2–4 °C and eluted with 100 mM NH$_4$HCO$_3$ at 15–25 mL/h. Fractions were collected and analyzed for protein and Au concentrations, from which the ratio of bound gold to BSA (Au/BSA) was determined. High and low molecular weight fractions were separately pooled, lyophilized, and redissolved in D$_2$O/NH$_4$HCO$_3$ buffer and CH$_3$OD, respectively, for ^{31}P NMR spectral measurements. **Method 2:** To 2.0 mL of AlbSAuPEt$_3$ (Au/BSA = 0.68; 1.06 mM BSA in 100 mM NH$_4$HCO$_3$ buffer) was added 1 equiv of KCN and 1.0 mL of CHCl$_3$. The mixture was stirred for 5 min and then the CHCL$_3$ layer was removed and evaporated to dryness. The residue was redissolved in CH$_3$OD for analysis by ^{31}P NMR.

Reaction of Et$_3$PAu^{13}CN with BSA. To 1.5 mL of BSA (1.97 mM in 100 mM NH$_4$HCO$_3$ buffer; SH/BSA = 0.60) was added 30 μL of a 332 mM methanolic solution of Et$_3$PAu^{13}CN (25% enriched). ^{31}P NMR spectra were accumulated, beginning immediately, for 20 min and then overnight, after which the ^{13}C spectrum was measured. The experiment was repeated with 1.78 mL of BSA (4.04 mM, SH/BSA = 0.66) and 47 μL of 100 mM Et$_3$PAuCN.

Reaction of [(Et$_3$P)$_2$Au]$^+$ with BSA. To 2.0 mL of BSA (2.08 mM, SH/BSA = 0.54) was added 100 μL of a 22.7 mM aqueous solution of [(Et$_3$P)$_2$Au]Cl. One milliliter of the reaction mixture was immediately transferred to a NMR tube containing 1.0 mL of D$_2$O. ^{31}P spectra were accumulated for 45 min and then overnight.

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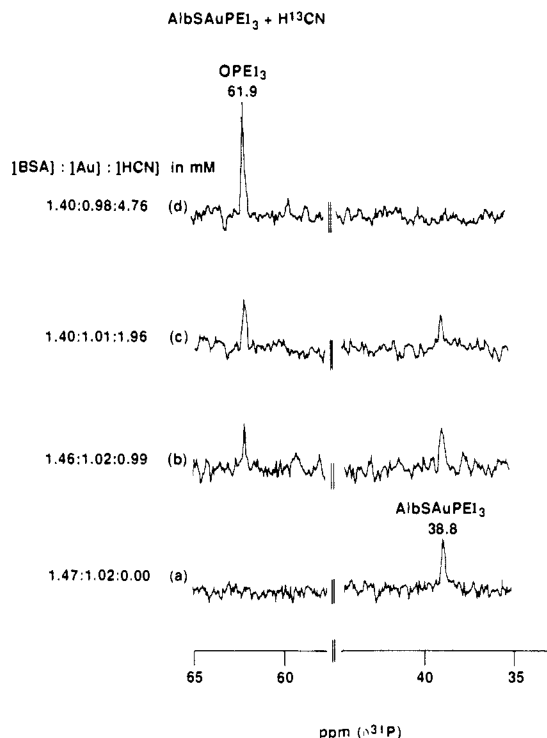


Figure 1. $^{31}P\{^{1}H\}$ NMR spectra (101.3 MHz) of $AlbSAuPEt_3$ titrated with various molar ratios of $H^{13}CN$. The concentrations of BSA, Au, and total cyanide are given in the figure. The $K^{13}CN$ used to prepare the stock solution immediately hydrolyzes to $H^{13}CN$ at pH 7.9.

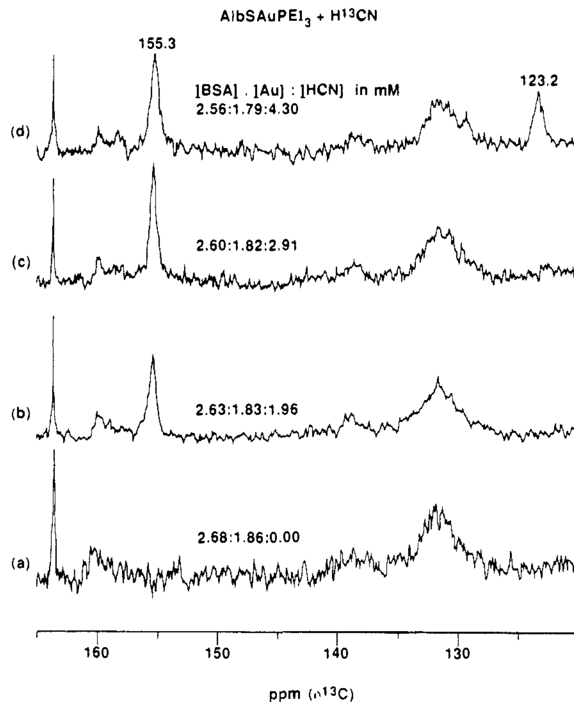


Figure 2. $^{13}C\{^{1}H\}$ NMR spectra (62.9 MHz) of $AlbSAuPEt_3$ sequentially titrated with various molar ratios of $H^{13}CN$. Concentrations are given in the figure.

Albumin Thiol Content. A solution of $AlbSAuPEt_3$ (0.76 Au/BSA; 0.77 mM BSA in 100 mM NH_4HCO_3 buffer) was prepared and analyzed for thiol content (<0.01 SH/BSA). To 500 μL of the $AlbSAuPEt_3$ was added 34 mL of KCN solution (10.0 mM). After 0.5 h the solution was chromatographed as above. Fractions were analyzed for albumin, gold, and thiol content. The Au–BSA ratio decreased to 0.11 and the SH/BSA ratio increased to 1.14.

Results

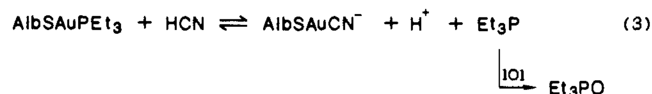
Irreversibly Formed Products from HCN + $AlbSAuPEt_3$. The $AlbSAuPEt_3$ for these studies was prepared by reacting bovine serum albumin of known mercaptalbumin (AlbSH) content with $Et_3PAuSATg$ and isolating the protein by chromatography.[12] Figure 1 shows the ^{31}P NMR spectra obtained by titrating $AlbSAuPEt_3$ with $H^{13}CN$. The KCN, which is actually added to the buffered, neutral solutions, immediately hydrolyzes to HCN and is discussed as such. As increasing amounts of $H^{13}CN$ are added, the 38.8-ppm resonance due to the $AlbSAuPEt_3$[12] decreases in intensity and the resonance at 61.9 ppm due to the Et_3PO[12] increases in intensity. These spectra clearly demonstrate that cyanide displaces Et_3P and, since no signal for Et_3P is observed, that Et_3P is very rapidly oxidized to Et_3PO. The presence of $AlbSAuPEt_3$ even after addition of 2 equiv of HCN (Figure 1c) results from the fact that the displacement reaction requires longer times than the ^{31}P spectral accumulations. By the time the final spectrum was accumulated (Figure 1d), no $AlbSAuPEt_3$ remained.

Figure 2 shows the ^{13}C NMR spectra for a similar titration of $AlbSAuPEt_3$ with $H^{13}CN$. The broad resonance at 132.0 ppm is from the BSA itself as shown in Figure 2a. The sharp resonance at 163.2 ppm is due to the CO_3^{2-} from the NH_4HCO_3 buffer. When approximately 1 equiv of $H^{13}CN$ was added (Figure 2b), a broad resonance at 155.3 ppm appeared. The position of this resonance is close to that of $[Au(CN)_2]^-$ (156.4 ppm) in aqueous solution, but it is broadened and slightly shifted. The shift and broadening are consistent with assignment as either $AlbSAuCN^-$, formed by displacement of the phosphine, or as $[Au(CN)_2]^-$ weakly bound to the protein. The $\Delta V_{1/2}$ of this resonance is 55 Hz. As the concentration of HCN increased, the resonance at 155.3 ppm increased in intensity (Figure 2c). However, above a ratio of $CN^-/Au = 2$, the resonance of free HCN appeared at 123.2 ppm (Figure 2d). This resonance has $\Delta V_{1/2} = 60$ Hz. The broadness of this peak indicates that this free HCN is in intermediate exchange with the cyano–gold species giving rise to the 155-ppm resonance. The ^{13}C NMR spectrum of the sample from Figure 1d is similar to that in Figure 2d, containing broad resonances at 155.3 and 122.0 ppm.

By analogy to reaction 2, $AlbSAuCN^-$ was expected to form by displacement of Et_3P from the protein–gold complex, $AlbSAuPEt_3$. The appearance of only a single, broad ^{13}C resonance at 155 ppm was ambiguous since $[Au(CN)_2]^-$ is also a possible product. Therefore, the amount of gold bound to albumin was determined by chromatographically isolating the protein after reactions between $AlbSAuPEt_3$ and increasing concentrations of HCN. In a typical set of reactions, the initial Au/BSA ratio was 0.69, and as the $H^{13}CN$/Au ratio was increased from 0.0 to 2.0, the Au/BSA ratio decreased to 0.25 ± 0.05. A ^{13}C NMR spectrum of the chromatographically isolated albumin no longer had the 155-ppm resonance. These results indicate that the resonance is due to $[Au(CN)_2]^-$, weakly bound to the albumin. The binding of the $[Au(CN)_2]^-$ to albumin explains why it does not exchange rapidly (on the NMR time scale) with the free HCN (Figure 2d), giving rise to a single averaged resonance, as normally occurs in aqueous solutions containing only HCN and $[Au(CN)_2]^-$.

The failure to observe the signal expected for $AlbSAu^{13}CN^-$ might be due to a short T_2 attributable to the long correlation time of the protein or to a subsequent reaction causing loss of the cyanide from the complex. To determine if cyanide was bound to the residual gold in the protein and silent in the NMR experiment, the chromatography experiments above were repeated with $K^{14}CN$ and measuring both the Au/BSA and $^{14}CN^-$/BSA ratios. Figure 3 shows the decrease in the Au/BSA ratios as the cyanide concentration increases. No cyanide is protein-bound at any point in the titration, indicating that $AlbSAuCN^-$ is not stable and does not accumulate as a product. The incubation time (20 min.) was short, but the decrease in the protein gold content demonstrated that the reaction was occurring.

The results of Figures 1, 2, and 3 can be described by eq 3, followed by either reaction 4a or 4b. Reaction 3 is analogous to the previously reported thiol reaction (reaction 2):[14]



$$AlbSAuPEt_3 + HCN \rightleftharpoons AlbSAuCN^- + H^+ + Et_3P \quad (3)$$
$$Et_3P \xrightarrow{[O]} Et_3PO$$

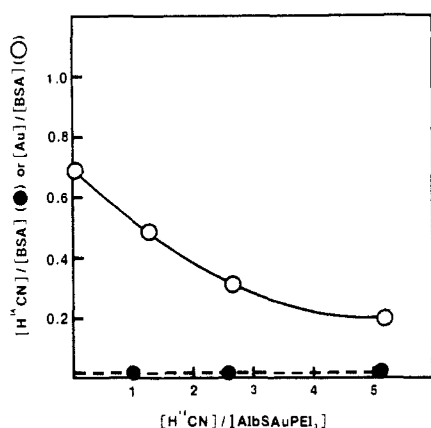


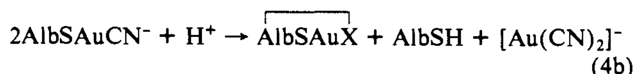
Figure 3. Chromatographic titration of AlbSAuPEt$_3$ (75.7 μM BSA; Au/BSA = 0.69) with H^{14}CN. The mixtures were incubated briefly and then passed over a small column of Sephadex G-50. Fractions were analyzed for gold, [H^{14}]cyanide, and albumin, from which the ^{14}CN/BSA and Au/BSA ratios were calculated.

The formation of $[Au(CN)_2]^-$ and the absence of AlbSAuCN$^-$ could result from a ligand scrambling reaction (reaction 4a) analogous to reaction 1:

$$2AlbSAuCN^- \rightleftharpoons (AlbS)_2Au^- + [Au(CN)_2]^- \quad (4a)$$

The location of the cysteine-34 residue in a crevice 10-Å deep[15] should disfavor the second product, a gold-bridged "dimer", (AlbS)$_2$Au$^-$. Nonetheless, the possibility of dimer formation[22] was tested by separating the products from a typical reaction over a very long gel-exclusion column (1.0 × 80 cm, Sephadex G-100) capable of resolving the gold-bridged dimer (128 000 daltons) from monomeric albumins (64 000 daltons). Equation 4a predicts that for the 17% conversion of gold to Au(CN)$_2^-$ observed, 34% of the albumin should have been converted to dimer. There was no increase in the albumin dimer population when the reaction products were compared to unreacted AlbSAuPEt$_3$. This finding is consistent with our previous negative[12,13] results on this question and sufficient to conclude that reaction 4a does not occur under these conditions.

An alternate pathway for the formation of $[Au(CN)_2]^-$ is reaction 4b:



$$2AlbSAuCN^- + H^+ \rightarrow \overline{AlbSAuX} + AlbSH + [Au(CN)_2]^- \quad (4b)$$

The large equilibrium formation constant for $[Au(CN)_2]^-$ provides a driving force for the reaction. The two-coordination requirement of the gold(I) remaining bound to the albumin (represented as $\overline{AlbSAuX}$ in reaction 4b) may be fulfilled by a second ligand from the protein. The reduction of albumin disulfides by triethylphosphine (vide infra) provides additional thiols which may function as ligands for the gold(I), but the participation of an appropriately located histidine or methionine cannot be discounted. The identification of the second ligand bound to the gold in $\overline{AlbSAuX}$ is beyond the scope of this present study. The formation and subsequent reaction of the postulated intermediate, AlbSAuCN$^-$ (reactions 3 and 4b), explain the absence of albumin-bound cyanide, the residual gold bound to albumin, and the formation of [Au(CN)$_2^-$].

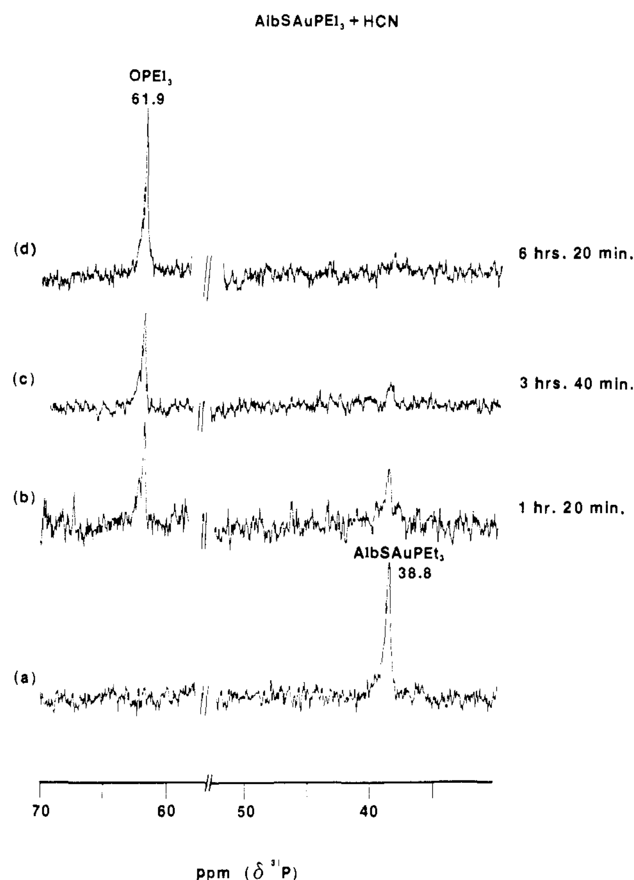


Figure 4. Time-dependent $^{31}P\{^1H\}$ NMR spectra (101.3 MHz) of an AlbSAuPEt$_3$ (2.05 mM BSA; 0.63 Au/BSA)/H^{13}CN (1.06 mM) reaction. (a) AlbSAuPEt$_3$ before cyanide addition; (b–d) spectra accumulated for the sequential time intervals indicated.

The spectra in Figures 1 and 2 indicate that cyanide generates Et$_3$PO more rapidly than the thiols examined previously.[14] The time scale for the cyanide reaction was determined by mixing the reactants and then immediately accumulating ^{31}P NMR spectra. When the cyanide is present in excess (CN$^-$/Au = 2.68), the reaction is complete within 10 min (not shown). When less than the stoichiometric quantity is used (CN$^-$/Au = 0.82), the reaction is nonetheless complete within 7 h (Figure 4).

Transient, Equilibrium Products in the HCN/AlbSAuPEt$_3$ Reaction. Et$_3$PAuCN was one of the products expected in the reaction of AlbSAuPEt$_3$ with HCN because the phosphine and cyanide are the ligands with the greatest affinities for gold(I). To determine whether Et$_3$PAuCN or other products might form and then react further, ^{31}P NMR spectra were recorded after isolating products from incomplete KCN/AlbSAuPEt$_3$ reactions. Two methods of separation were used. In the first method, equimolar KCN and AlbSAuPEt$_3$ (Au/BSA = 0.59) were mixed and then immediately separated by gel-exclusion chromatography. During the very short reaction time as separation occurred ($\leq$10 min), the Au/BSA ratio decreased from 0.59 to 0.36. For the pooled and concentrated high molecular weight fractions (Figure 5a), resonances due to Et$_3$PO (61.9 ppm)[12] and AlbSAuPEt$_3$ (38.8 ppm)[12] were observed. For the low molecular weight (LMW) components, lyophilized and redissolved in methanol (Figure 5b), resonances were detected for Et$_3$PO (57.3 ppm)[12] and Et$_3$PAuCN (35.4 ppm).[11]

The second method applied to isolate the transient products was extraction of the AlbSAuPEt$_3$/KCN mixture with CHCl$_3$. The Au/BSA decreased from 0.68 to 0.19. The ^{31}P NMR spectrum (Figure 5c) of the extractable, nonpolar components redissolved in CH$_3$OD displayed resonances for Et$_3$PAuCN (35.5 ppm)[11] and [(Et$_3$P)$_2$Au]$^+$ (45.7 ppm).[11,18] Only a small amount of Et$_3$PO (57.1 ppm)[12] was extracted since it is quite water soluble.

(22) Although our previous experience with albumin gold complexes led us to discount the possibility of gold-bridged dimers, a referee invoked an analogy to albumin dimers which form as artifacts of albumin isolation procedures. These dimers probably form via disulfide interchange reactions of Cys-34 from one albumin and a disulfide from a second albumin. No evidence for a Cys-34 to Cys-34 linkage among the three discrete and resolvable albumin dimers (ref 23) is known to the present authors. Likewise, the often cited Hg-bridged dimers precipitate slowly from H$_2$O/ethanol over several days (ref 17) and may involve an intramolecular disulfide interchange reaction preceding dimer formation. The ligand exchange reactions studied here are complete in 10 min to 10 h.

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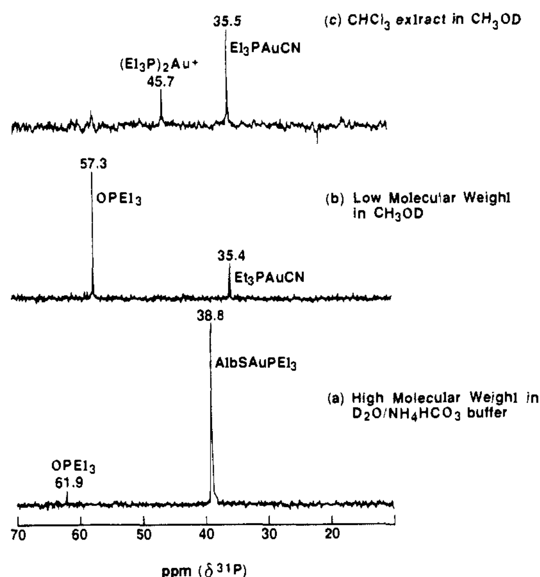


Figure 5. $^{31}P\{^1H\}$ NMR spectra identification of the transient, kinetic product, Et_3PAuCN. (a) The high molecular weight and (b) the low molecular weight components of an equimolar KCN + $AlbSAuPEt_3$ (1.69 mM BSA; Au/BSA = 0.59) reaction mixture separated by gel chromatography immediately after mixing the reactants. (c) Non-protein reaction components isolated by $CHCl_3$ extraction from a similiar reaction (1.06 mM BSA, Au/BSA = 0.68). $[(Et_3P)_2Au]^+$ forms by ligand disproportionation (reaction 1).

Both methods confirmed the presence of Et_3PAuCN as a transient product. Its formation can be explained via a simple ligand exchange equilibrium:

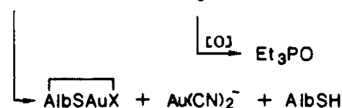
$$AlbSAuPEt_3 + HCN \rightleftharpoons Et_3PAuCN + AlbSH \quad (5)$$

The chloroform extraction procedure also demonstrated that $[(Et_3P)_2Au]^+$ is present in equilibrium with Et_3PAuCN according to reaction 1. The absence of the $[(Et_3P)_2Au]^+$ in the spectrum of the chromatographically isolated LMW products (Figure 5b) can be explained by the presence of additional $[Au(CN)_2]^-$, produced via reaction 4b, which represses the equilibrium of reaction 1.

Reaction of Et_3PAuCN with AlbSH. To verify that the transient, equilibrium product Et_3PAuCN can react with the protein to form Et_3PO, the reactions of 25% ^{13}C-enriched Et_3PAuCN with AlbSH at 5:1 and 1:1 ratios were monitored by ^{31}P NMR (not shown). For the 5:1 reaction mixture, the spectrum accumulated for the first 20 min contained two resonances, 38.8 ppm due to $AlbSAuPEt_3$[12] and 62.0 ppm due to Et_3PO.[12] The phosphine oxide represented approximately 90% of the total phosphorous and the protein complex about 10%, indicating that the oxide forms rapidly. The spectrum which was accumulated for the next 12 h showed both the Et_3PO and the $AlbSAuPEt_3$ resonances but the latter had decreased in intensity. The ^{13}C NMR spectrum accumulated at the end of the 12 h detected $Au(CN)_2^-$ weakly bound to the albumin (155 ppm) as the only cyanide species. The ^{31}P NMR spectrum of the 1:1 reaction mixture accumulated for several hours after mixing displayed resonances for the Et_3PO and $AlbSAuPEt_3$, corresponding to 62 and 38%. Thus, Et_3PAuCN and albumin react to form $AlbSAuPEt_3$ and HCN as transient, equilibrium products (reaction 6) and the same ultimate (irreversibly formed) products described in reactions 3 and 4b.

$$Et_3PAuCN + AlbSH \rightleftharpoons AlbSAuPEt_3 + HCN \quad (6)$$

$$Et_3PAuCN + AlbSH \longrightarrow AlbSAuCN^- + H^+ + Et_3P \quad (7)$$

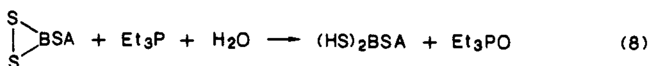


$$Et_3P \xrightarrow{[O]} Et_3PO$$

$$AlbSAuCN^- \longrightarrow \overline{AlbSAuX} + Au(CN)_2^- + AlbSH$$

Reaction 6 is the reverse of reaction 5, demonstrating that they are indeed reversible, equilibrium reactions as formulated. The intermediate $AlbSAuCN^-$ reacts further according to reaction 4b.

Reduction of Albumin Disulfide Bonds. The oxidation of Et_3P to Et_3PO has been previously postulated to occur by reduction of the albumin disulfide bonds.[14,18] Therefore, DTNB was employed to determine whether the thiol content of albumin was increased. Reaction mixtures similar to those in Figure 3 were chromatographed to remove unreacted cyanide, which could interfere with the measurement, and then analyzed with DTNB. The thiol titer of the $AlbSAuPEt_3$ before the reaction was 0.01, because the gold masks the reactivity of Cys-34. During the reaction, the gold content decreased from 0.69 to 0.11, while the thiol titer (SH/BSA) increased to 1.14. Since this value is significantly greater than the original SH titer (0.58), it indicates significant reduction of disulfide bonds to thiols during the reaction. This aspect of the reaction is not restricted to mercaptalbumin, but presumably it includes all albumin species (BSA):



$$S_2BSA + Et_3P + H_2O \longrightarrow (HS)_2BSA + Et_3PO \quad (8)$$

Reaction of $[(Et_3P)_2Au]^+$ and AlbSH. The ligand-scrambling reaction of Et_3PAuCN (reaction 1), described previously,[11] should generate $[(Et_3P)_2Au]^+$ in the reaction medium. Large excesses of this cationic complex are known to denature albumin with concomitant formation of Et_3PO.[18] Therefore, the reaction of equimolar $[(Et_3P)_2Au]^+$ and albumin was examined by ^{31}P NMR spectroscopy. The spectrum accumulated immediately after mixing the reactants contained resonances due to $[(Et_3P)_2Au]^+$ (44.3 ppm), Et_3PO (61.9 ppm),[12] and $AlbSAuPEt_3$ (38.5 ppm),[12] indicating that reaction 9 occurs:

$$[(Et_3P)_2Au]^+ + AlbSH \longrightarrow AlbSAuPEt_3 + Et_3P \quad (9)$$

$$Et_3P \xrightarrow{[O]} Et_3PO$$

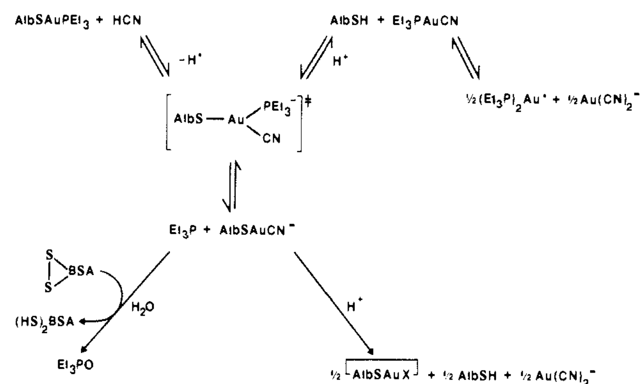
After 14 h, the resonance for the $[(Et_3P)_2Au]^+$ disappeared and there was a new, weak, broad resonance at 35.8 ppm in addition to the Et_3PO and $AlbSAuPEt_3$ resonances. The resonance is in the chemical shift range characteristic of thiolate complexes, Et_3PAuSR.[12,14] It is reasonably assigned to a $(Et_3PAuS)BSA$ complex in which Et_3PAu^+ binds to a sulfhydryl group other than Cys-34. New sulfhydryl groups are generated from disulfide bonds as Et_3P is oxidized to Et_3PO (reaction 8). Thus, this 35.8-ppm resonance provides further experimental evidence for the reduction of disulfide bonds by Et_3P.

Discussion

An associative mechanism for the displacement of triethylphosphine from gold(I) by a thiol was proposed to explain the slow formation of Et_3PO when $AlbSAuPEt_3$ is exposed to glutathione or other thiols.[14] This concept contravenes the usual expectation that phosphines are better ligands for gold(I) than thiols. Et_3P, however, is rapidly oxidized to Et_3PO, a poor Lewis base for soft metals, thereby shifting the equilibrium toward phosphine loss.[14] A testable prediction of the mechanism is that replacing the thiol with cyanide, which has a greater affinity for gold(I) than does Et_3P, should accelerate the oxidation of phosphine. Figures 1 and 4 present convincing data for extensive formation of Et_3PO over 3 to 7 h. In contrast, the reactions with thiols (reaction 2) are incomplete even after 24 h.[14] The greater thermodynamic affinity of the cyanide for gold(I) causes the equilibrium of reaction 3 to lie much further to the right than the corresponding reactions of thiols (reaction 2). The consequent increase in the concentration of Et_3P results in more rapid oxidation to Et_3PO.

An unusual aspect of these albumin/phosphine/cyanide/gold(I) reactions is that two sets of reactants ($AlbSAuPEt_3$ + HCN or Et_3PAuCN + AlbSH) can generate the same set of ultimate (i.e., irreversibly formed) products, Et_3PO, $[Au(CN)_2]^-$, AlbSH, and $\overline{AlbSAuX}$. In either case the other set of reactants are transient, equilibrium products. Scheme I summarizes the results of reactions 3, 4b, and 5–8. A three-coordinate transition state, analogous to that proposed previously for the thiol-induced formation of Et_3PO from $AlbSAuPEt_3$,[14] elegantly links either set

Scheme I



of reactants to its equilibrium products and to the irreversibly formed products.

Thus, when HCN reacts with AlbSAuPEt$_3$, the transient products, Et$_3$PAuCN and mercaptalbumin (AlbSH), form in equilibrium with the starting materials. Similarly, Et$_3$PAuCN and AlbSH transiently form AlbSAuPEt$_3$ and HCN. The two sets of equilibrating reactants are shown at the top of the scheme. In each case, the reaction proceeds to form, essentially irreversibly, the stable products shown at the bottom of the scheme. The third set of conceivable ligand exchange products, Et$_3$P and AlbS-AuCN$^-$, are proposed to be intermediates leading from the three-coordinate transition state to the irreversibly formed products. This transition state is consistent with the known preference of gold(I) to react via associative pathways for ligand exchange.[24-27] Numerous examples of stable three-coordinate gold(I) complexes have been characterized[28] and other d^{10} metal systems also react via associative mechanisms.[29]

Before proceeding, the possibility of other mechanisms should be considered. The most likely alternative for ligand exchange is a rate-limiting first-order dissociation of the phosphine from AlbSAuPEt$_3$, followed by a rapid, irreversible oxidation to Et$_3$PO. Such a mechanism would predict that the reaction rate would be independent of the nature of any ligands present and that the reaction should proceed even in the absence of any ligand. These predictions are inconsistent with the absence of Et$_3$PO formation when AlbSAuPEt$_3$ alone is in solution and when Et$_3$PAuCl and AlbSH react forming AlbSAuPEt$_3$ and chloride, which is too weak a ligand to displace phosphine.[12] Further, it cannot explain why the rate of phosphine formation in the presence of thiols increases as the affinity of the thiol for gold(I) increases.[14] Nor would it account for the observation reported here that cyanide greatly accelerates the oxidation of Et$_3$P. A dissociative mechanism has been proposed for the thermal decomposition of CH$_3$AuPΦ_3,[30] but the nature of the reaction and its products (especially ethane) are quite different from those proposed here. The free radical[31] and oxidative addition–reductive elimination[32] mechanisms proposed for other reactions generating hydrocarbons from alkyl-gold complexes and the S_{E2} mechanism for the transfer of a methyl group from gold to mercury[33] are not applicable to the simpler ligand exchanges observed here.

The initial equilibria among the three sets of reactants/products (HCN + AlbSAuPEt$_3$; AlbSH + Et$_3$PAuCN; or AlbSAuCN$^-$ + Et$_3$P) will depend on the affinities of the ligands for gold(I), their leaving group abilities (which in aqueous solution will be determined partly by solvation effects) and the aqueous solubilities of the small gold complexes involved. The affinity of the ligands for gold(I) is CN$^-$ $\gg$ Et$_3$P > AlbSH. That order should favor the upper right set of products. The albumin Cys-34 thiol, compared to other thiols, has an unusually high affinity for gold(I), as indicated by its low pK_{SH} value[34] and the large chemical shift of AlbSAuPEt$_3$.[14] Thus, albumin competes with and displaces phosphine or cyanide from gold(I), leading to the transient intermediates and final products. The initial proportions of species resulting from these complex equilibria are short-lived due to the irreversible oxidation of the liberated phosphine to Et$_3$PO.

As the reaction proceeds, some but not all of the gold initially bound to albumin appears as [Au(CN)$_2$] (Figures 2 and 3). Its formation is consistent with the large equilibrium binding constant, log β_2 = 36.6,[8] of the dicyano–gold(I) complex. Figure 3, however, shows that its formation according to reaction 4 is less complete than might be expected from the magnitude of β_2, since even at a cyanide-to-gold ratio exceeding 2 the reaction is incomplete. This anomaly can also be explained by the binding of gold to the newly generated thiols and the high affinity of the albumin Cys-34 thiol for gold(I).

The ligand scrambling equilibrium of Et$_3$PAuCN (reaction 1) generates [(Et$_3$P)$_2$Au]$^+$,[11] which in large excess denatures albumin, simultaneously forming Et$_3$PO.[15] Under the conditions used here, the reaction of equimolar [(Et$_3$P)$_2$Au]$^+$ with albumin leads to rapid formation of Et$_3$PO and AlbSAuPEt$_3$. Thus, a second competing pathway for Et$_3$PO formation (not shown in the scheme) is the reaction of [(Et$_3$P)$_2$Au]$^+$ with AlbSH (reaction 9). The relative contribution of this alternate pathway depends on the concentrations of Et$_3$PAuCN and [(Et$_3$P)$_2$Au]$^+$ and on the rates at which AlbSH displaces phosphine from each complex.

The increase in the thiol titer of albumin during the reaction of HCN with AlbSAuPEt$_3$ provides direct evidence that the disulfide bonds of albumin are reduced by triethylphosphine (reaction 8). Further evidence for the reduction of the albumin disulfide bonds is provided by the appearance of the 35.8-ppm resonance in the ^{31}P NMR spectrum of the reaction of [(Et$_3$P)$_2$Au]Cl with BSA. We attribute this resonance to the binding of Et$_3$PAu$^+$ to the new sulfhydryl groups of albumin to form (Et$_3$PAuS)BSA. Thus, the role of the albumin disulfides as the oxidants for triethylphosphine oxide formation in the presence of serum albumin, as originally proposed by Sadler,[18] receives direct experimental support from the data reported here. In a related study,[19] we demonstrated that, as required by reaction 8, oxygen from H$_2$^{17}O is incorporated into ^{17}OPEt$_3$ when glutathione and AlbSAuPEt$_3$ react in H$_2$^{17}O.

The chemistry described here may affect the gold metabolism of chrysotherapy patients who smoke. Hydrogen cyanide is present in tobacco smoke at a concentration of 1600 ppm[35] and is absorbed into the blood. In the absence of gold, it undergoes two reactions: reversible binding to met-hemoglobin in red blood cells and irreversible oxidation to thiocyanate.[36] As a consequence of these reactions, the concentration of free HCN in the blood of smokers (ca. 20 μM) is only slightly greater than that of nonsmokers (ca. 12 μM). Significantly increased levels of thiocyanate in smokers, however, indicate that substantial quantities of HCN enter the blood stream and are metabolized. In smoking patients receiving aurothiomalate or other oligomeric drugs, hydrogen cyanide transports gold metabolites into red blood cells.[2,3] Since these gold metabolites normally do not enter rbc's, the gold–cyanide reactions must compete effectively with the usual in vivo reactions of HCN. Thus, the gold may act as a cyanide-sink and in the process undergo an altered metabolism.

A substantial concentration of gold is normally present in rbc's after auranofin administration,[7] and smoking does not substantially

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increase it.[3] The chemistry of the HCN/AlbSAuPEt$_3$ reaction described here suggests, however, that HCN from inhaled smoke may alter auranofin metabolism more subtly by accelerating the formation of Et$_3$PO in vivo. The acetylthioglucose ligand of auranofin is rapidly displaced and metabolized, while the gold-bound phosphine is slowly displaced and oxidized over 24 to 72 h.[7] If, as HCN enters the blood, it reacts with AlbSAuPEt$_3$ (which forms in whole blood, in serum, and in direct reactions of auranofin with albumin), the displacement and subsequent oxidation of the phosphine should occur more rapidly in smokers than in nonsmokers. The extent to which accelerated Et$_3$PO production is observed in vivo will depend on the competition between cyanide binding to gold and its usual metabolic fates: binding to methemoglobin and oxidation to thiocyanate.

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Registry No. HCN, 74-90-8; Et$_3$PAuCN, 90981-41-2; Et$_3$P, 554-70-1; Et$_3$PO, 597-50-2; [(Et$_3$P)$_2$Au]$^+$, 45154-29-8.

Evidence for an Episulfonium Ion Intermediate in the Formation of S-[2-(N^7-Guanyl)ethyl]glutathione in DNA

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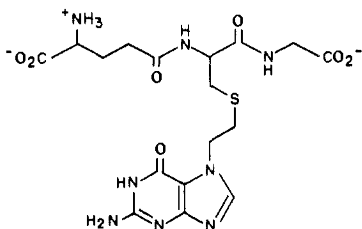
Abstract: The carcinogen ethylene dibromide (EDB) is bioactivated via a pathway involving initial conjugation with the tripeptide glutathione (GSH) in a reaction catalyzed by GSH S-transferase. The conjugate then reacts preferentially with DNA guanyl residues to generate S-[2-(N^7-guanyl)ethyl]glutathione. Rates of hydrolysis and alkylation of 4-(p-nitrobenzyl)pyridine with several cysteinyl and homocysteinyl analogues of S-(2-haloethyl)glutathione at pHs 2.2, 6.4, and 8.5 are consistent with the hypothesis that an episulfonium ion is a common intermediate in both the hydrolysis and alkylation reactions. Consistently, 2-amino-6-chlorohexanoic acid failed to react with 4-(p-nitrobenzyl)pyridine. The stereochemical course of the overall reaction was studied with [threo-1,2-$^2H_2$]EDB and [erythro-1,2-$^2H_2$]EDB, which were incubated with GSH, rat liver cytosol, and DNA; the resulting DNA N^7-guanyl adducts were isolated and analyzed by NMR techniques in order to determine the stereochemical course of the reaction. Two-dimensional correlated (COSY) NMR indicated that the reaction had occurred by a single stereochemical course. The magnitude of nuclear Overhauser effects between the ethylene protons suggests that the reaction occurs with net inversion of configuration of the methylene protons. This conclusion was confirmed upon comparison of the COSY NMR spectra of the biologically generated adducts with those that were synthetically prepared from the deuterated EDB diastereomers via a known stereochemical route. This observation, combined with the kinetic data, supports a reaction mechanism where the EDB–GSH conjugate forms an episulfonium ion prior to reaction with DNA guanyl residues.

Ethylene dibromide (EDB), an extensively used agricultural and industrial chemical, is mutagenic in a variety of test systems[1-4] and is carcinogenic in laboratory animals.[4-8] While epidemiological studies on humans exposed to large EDB doses are inconclusive with regard to carcinogenic risk,[9,10] EDB is acutely toxic and has caused two human deaths.[11] Recent public concern about carcinogenicity has led to the ban of its use as a pesticide.[12]

While EDB is primarily metabolized via an oxidative cytochrome P-450 initiated route,[13,14] the majority of the experimental evidence supports the hypothesis that the pathway responsible for the genotoxicity of this and other vic-dihaloethanes involves glutathione S-transferase catalyzed conjugation with the tripeptide glutathione (GSH) to yield S-(2-bromoethyl)glutathione (route a, Figure 1).[1,14-21] The conjugate reacts preferentially with DNA[16] to form primarily S-[2-(N^7-guanyl)ethyl]glutathione.[15,19,22]



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[§] Department of Chemistry.

The precise nature of the ultimate DNA alkylating species is unknown. The instability of the putative S-(2-bromoethyl)glu-

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