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CELLULAR INTERACTIONS OF AURANOFIN AND A RELATED GOLD COMPLEX WITH RAW 264.7 MACROPHAGES

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Abstract—Auranofin (AF) is an orally active chrysotherapeutic agent whose precise mechanism of action with its putative target cell, the macrophage, is not known. In a previous paper, we described a sequential thiol exchange mechanism that explained auranofin's molecular mechanism of interaction with RAW 264.7 cells. To further understand the mode of action of AF and to test the validity of the thiol exchange model, we have continued to study the interactions with macrophages of AF and a related gold complex, triethylphosphine gold chloride (TEPG). Evaluation of the effects of AF and TEPG on RAW 264.7 cells demonstrated that: (1) more gold from TEPG than AF associated with cells over time and with a variety of concentrations; and (2) cellular association of AF and TEPG was temperature dependent. The energy of activation for cell association, the rate-limiting step in the thiol exchange process, was lower for TEPG than AF; (3) cellular association and uptake of both compounds did not require metabolic energy; and (4) efflux of both AF and TEPG was time, temperature, and thiol dependent. Based on these and previous data, we conclude that sequential thiol exchange may be a generic phenomenon for cellular uptake and distribution of thiol reactive gold compounds and that the rate-limiting step is the exchange of either tetraacetylthioglucose (TATG) or chloride for a membrane-localized sulfhydryl group.

Auranofin, AF† (Fig. 1) [(1-thio-β-D-glucopyranose 2,3,4,6-tetraaceto-*S*)(triethylphosphine)gold], is an orally active chrysotherapeutic agent used for the treatment of rheumatoid arthritis [1–3]. Recent evidence suggests that its primary therapeutic target is the macrophage, a cell that plays a critical role in the initiation and maintenance of chronic rheumatoid synovitis [4–7]. Although AF has been shown to modulate many *in vivo* and *in vitro* activities of macrophages, including chemotaxis, phagocytosis, interleukin-1 and superoxide production, its mechanism of action with these cells is still unknown [6–9]. In addition, little is known concerning the mechanisms of AF's cellular association, uptake and intracellular distribution.

Based on data obtained using AF radiolabeled within the triethylphosphine (Et_3P) [^3H], the gold [^{195}Au] or the tetraacetylthioglucose (TATG) [^{14}C] moieties of the molecule, we proposed a model, a sequential thiol exchange mechanism that describes the metabolism and subsequent molecular interactions of AF with RAW 264.7 cells (Fig. 2, panels A, B and C) [10]. In this model, cell association (Fig. 2A), or the net cellular accumulation of the drug, results from the sequential shuttling of the Au-Et_3P or Au moieties of the AF molecule between cellular sulfhydryl groups; initial cell association, the rate-limiting step in the sequential exchange process, occurs because membrane-localized thiols compete with the TATG moiety of AF for one of the coordinate bonds of gold.

A secondary competing reaction may also occur in which extracellular thiols such as albumin or cysteine compete with membrane-localized thiols for the TATG-ligating site. Intracellular distribution of AF (Fig. 2B) results from shuttling of membrane sulfhydryl bound gold-triethylphosphine to cytosolic sulfhydryl groups. Although only a single sulfhydryl exchange is shown, multiple intramembrane ligand exchanges may take place before final cytosolic sulfhydryl association. Alternatively, oxidative loss of the triethylphosphine moiety in the membrane may precede AF gold shuttling into the cytoplasm. Cellular efflux (Fig. 2C) is simply the reverse of cell association and intracellular distribution.

To better understand the mechanism of action of AF and to test the validity of the thiol exchange model, we have continued to investigate its effects on RAW 264.7 macrophage-like cells [11]. In addition, we have examined the cellular uptake, distribution and efflux of triethylphosphine gold chloride (TEPG) (Fig. 1), the chloro analog of AF that also possesses antiarthritic activity [12–14], and compared these data to those obtained with AF. Results of our studies allow us to confirm [10] that

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† Abbreviations: AF, auranofin [(1-thio-β-D-glucopyranose2,3,4,6-tetraacetato-*S*)(triethylphosphine)gold]; Et_3P, triethylphosphine; TATG, tetraacetylthioglucose; TEPG, triethylphosphine gold chloride; FCS, fetal calf serum; DMEM, Dulbecco's low glucose minimal essential medium; DNP, 2,4-dinitrophenol; PBS, phosphate-buffered saline; E_a, energy of activation; GSH, glutathione; and NEM, *N*-ethylmaleimide.

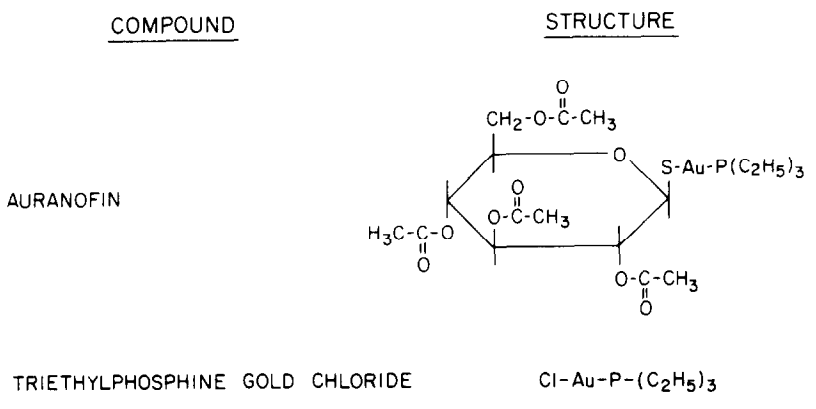

Fig. 1. Structures of auranofin [(1-thio-β-D-glucopyranose2,3,4,6-tetraacetato-*S*)(triethylphosphine)gold] and triethylphosphine gold(I) chloride. The compounds were labeled with [^{195}Au] at the gold moieties of both molecules.

sequential thiol exchange may be a generic phenomenon for cellular uptake and distribution of thiol reactive gold compounds, and that the rate-limiting step is the exchange of either the TATG or chloride for a membrane-localized sulfhydryl group.

MATERIALS AND METHODS

Materials. [^{195}Au]Auranofin (4.6 mCi/mmole) and [^{195}Au]triethylphosphine gold chloride (4.6 mCi/mmole) were obtained from DuPont, New England Nuclear (Boston, MA), and supplied to us by Dr. Richard Heyes. Pentex reagent grade bovine albumin, fraction V, was obtained from Miles Scientific (Naperville, IL), and Dulbecco's low glucose minimal essential medium (DMEM) and fetal calf serum (FCS) were purchased from the Grand Island Biological Co. (Grand Island, NY). *N*-Ethylmaleimide (NEM) was obtained from Baker (Phillipsburg, NJ). All other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO).

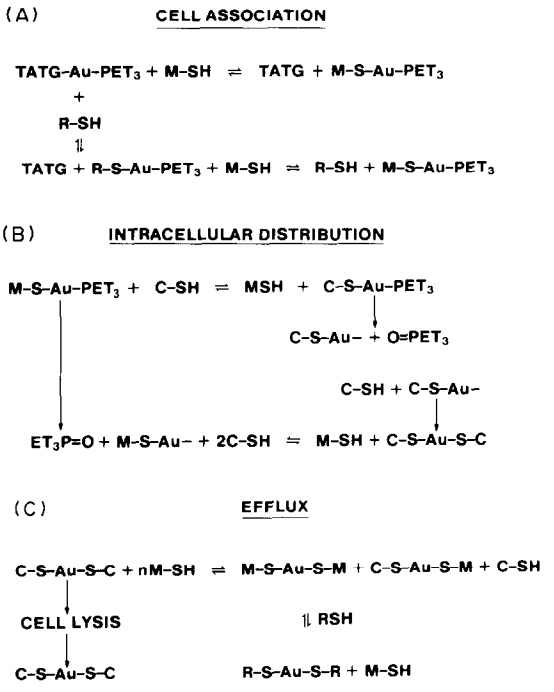

Fig. 2. Model of cell association (A), intracellular distribution (B), and efflux (C), of AF and metabolites. M-SH: membrane-localized sulfhydryl groups; R-SH: extracellular sulfhydryl groups; C-SH: cytosolic sulfhydryl groups; Et_3P: triethylphosphine; and TATG: tetraacetylthioglucose.

Cell culture techniques. RAW 264.7 cells, a murine macrophage-like cell line obtained from the American Type Culture Collection (ATCC TIB 71), were grown in monolayer in DMEM containing 10% FCS in a 5% CO_2 humidified incubator at 37°. The cells were determined to be mycoplasma-free by the Hoescht 33258 procedure [15].

Cell association of [^{195}Au]*AF and* [^{195}Au]*TEPG.* Asynchronous, confluent populations of RAW 264.7 cells were scraped from T-150 mm flasks and resuspended in fresh DMEM to achieve a final concentration of 1.2×10^6 cells/ml. Medium containing 100 μl of [^{195}Au]-labeled AF and TEPG was added to 900 μl of cell suspension. Cells and the radiolabeled compounds were incubated under conditions described in Results. The reactions were stopped by placing the tubes on ice and centrifuging the reaction mixture at 4° in a Beckman Tabletop Centrifuge for 5 min at 3000 rpm. Both supernatant fraction and cell pellet were placed in scintillation vials, and radioactivity was determined in a Beckman Gamma 8000 Counter.

Cells (1×10^6 cells/ml) were also treated with increasing concentrations of 2,4-dinitrophenol (DNP), NEM, and NaF for 5, 30 and 60 min respectively. The reactions were stopped by centrifuging the reaction mixture at 4° for 5 min at 3000 rpm. After centrifugation, the supernatant fraction was removed from the tubes, and the cell pellets were washed three times with ice-cold PBS. After recentrifugation for 5 min at 4° at 3000 rpm, the supernatant fraction was again removed from the centrifuge tubes. The cells were then resuspended in 900 μl fresh DMEM and 100 μl [^{195}Au]AF or [^{195}Au]TEPG and incubated for 10 min at 37° in a shaking water bath. The reactions were stopped, and

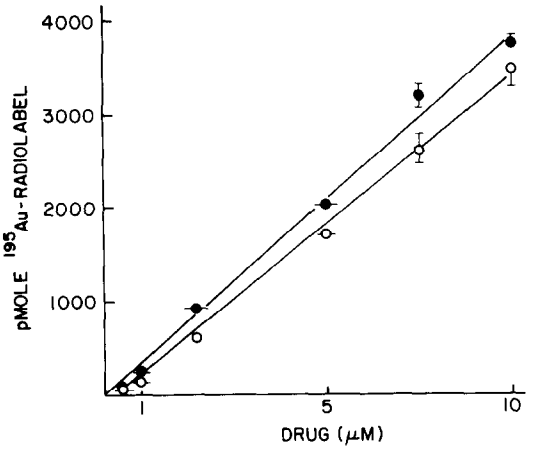

Fig. 3. Cellular association of [^{195}Au]radiolabel derived from AF and TEPG as a function of increasing concentrations of each drug. Procedures are as described in Materials and Methods. Values shown are means and standard deviations from two separate experiments performed in triplicate. Key: (○—○) [^{195}Au]AF; and (●—●) [^{195}Au]TEPG.

radioactivity was determined as described in the previous section.

Determination of initial rates of [^{195}Au]*AF and* [^{195}Au]*TEPG cell association.* The rates of cellular association of AF and TEPG were treated as in [10] as simple pseudo-first-order processes with respect to cellular sulfhydryl groups, a treatment which seems reasonable in view of the estimated sulfhydryl group concentration in cellular membranes (10^{-8}–10^{-11} M) [16] and the large excess of AF and TEPG used. The experimental data were fit by a standard multiple parameter, least squares analysis to a semilogarithmic plot which provides pseudo-first-order rate constants directly from the slope of the resulting linear expression [10].

The energy of activation (E_a) for cellular association for AF and TEPG was determined by plotting the log of the initial rate versus the inverse of the temperature (°K).

Dissociation of [^{195}Au]*AF and* [^{195}Au]*TEPG.* [^{195}Au]AF (5 μM) and [^{195}Au]TEPG (5 μM) were incubated with 1×10^6 macrophages for 20 min in a 37° shaking water bath (New Brunswick Scientific). The cells were then centrifuged at 3000 rpm × 5 min, washed once with PBS, and recentrifuged, and then the supernatant fraction was removed. The cells were resuspended in 1.0 ml DMEM alone or DMEM containing FCS, albumin or glutathione. After a second incubation of either 4° or 37° for specified times, the reaction mixture was centrifuged at 3000 rpm for 5 min and the supernatant fraction was removed. The cell pellet and supernatant fraction were transferred to scintillation vials, and radioactivity was determined in a Beckman Gamma Counter.

RESULTS

Association of [^{195}Au]*AF and* [^{195}Au]*TEPG with RAW 264.7 cells.* Figure 3 compares the amount of [^{195}Au]-labeled AF and TEPG that associated with cells after a 30-min incubation at 37°. Cellular association of both drugs was linearly proportional with no evidence of saturation at concentrations as high as 10 μM. Under identical experimental conditions, more [^{195}Au] from TEPG than AF was associated with these cells. Incubation of cells with concentrations of the drugs greater than 7.5 μM resulted in similar pmole incorporation of the gold.

The time course of association for [^{195}Au]AF and [^{195}Au]TEPG (5 μM) is shown in Fig. 4. The cell-associated [^{195}Au] from both compounds increased as a function of time for 10 min and then plateaued between 10 and 30 min. After 30 min the amount of cell-associated [^{195}Au] decreased, as did cell number, resulting perhaps from the cytotoxic effects of both compounds. At all times, except 60 min, more

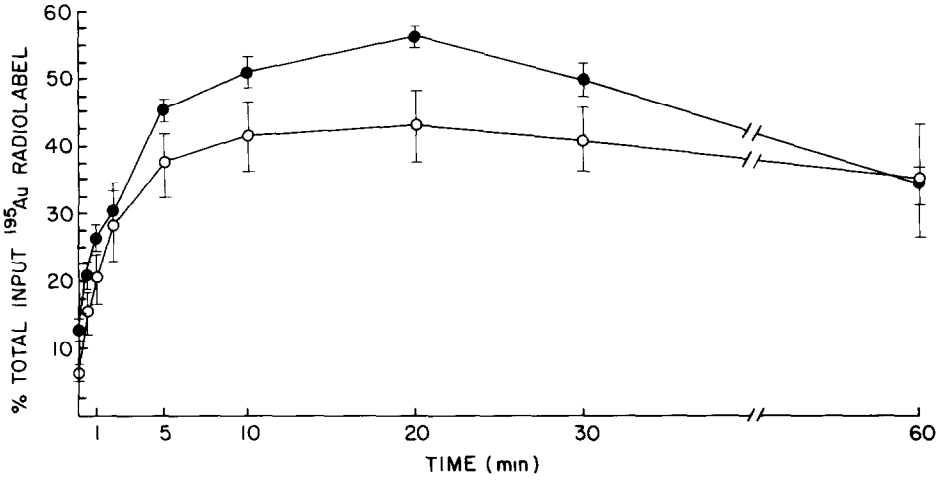

Fig. 4. Cellular association of [^{195}Au] from 5 μM AF and TEPG as a function of time. Procedures are as described in Materials and Methods. Values shown are means and standard deviations from two separate experiments performed in triplicate. Key: (○—○) [^{195}Au]AF; and (●—●) [^{195}Au]TEPG. For both AF and TEPG, 100% equals 30,000 to 35,000 cpm/ml.

Table 1. Auranofin and triethylphosphine gold chloride initial rate constants (K_{obs}) at increasing incubation temperatures

Temperature	K_{obs} AF	K_{obs} TEPG
15°	0.047	0.120
22°	0.088	0.170
27°	0.148	0.180
30°	0.158	0.220
37°	0.461	0.410

Cells (1×10^6 cells/ml) were treated with 5 μM [^{195}Au]-radiolabel. The percent of total input radioactivity associated with cells was determined at various times and temperatures as described in Materials and Methods. The experimental data was fit by a standard multiple parameter, least squares analysis to a semilogarithmic plot which provides pseudo-first-order rate constant (K_{obs}) directly from the slope of the resulting linear expression.

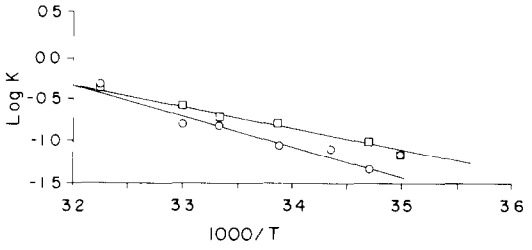

Fig. 5. Arrhenius plot for determination of E_a for AF and TEPG. Procedures are as described in Materials and Methods. The log of the initial rate of cell association (log K) was determined at each temperature as described in Table 1. Key: (○—○) AF; and (□—□) TEPG.

[^{195}Au] from TEPG than AF was associated with cells.

We previously reported [10] that association and uptake of [^{195}Au] from labeled AF is temperature dependent from 4 to 37°. As with AF, cellular association of [^{195}Au]TEPG was also temperature dependent (data not shown). Table 1 compares the initial rates of cell association for both compounds. The data demonstrate that the initial rates of cell association increased as the temperature increased. In general, these rates were higher for TEPG than AF. The Arrhenius plot derived from initial rates of cell association is shown in Fig. 5. The energy of activation for TEPG was 9.77 kcal/mole, while that for AF was 16.8 kcal/mole. The higher initial rates (K_{obs}) and lower E_a of TEPG compared to AF probably resulted from the fact that chloride is a better leaving group than the TATG moiety of the AF molecule [17].

Cellular association of [^{195}Au]*AF and* [^{195}Au]*TEPG after pretreatment with NEM, DNP and* NaF. The data in Table 2 show that cellular association and uptake of [^{195}Au]radiolabel from AF and TEPG were equally inhibited by a 30-min pretreatment of cells with NEM [18], an irreversible sulfhydryl alkylating agent, with 1000 μM NEM resulting in 83 and 85% reduction in [^{195}Au] association relative to control for TEPG and AF respectively. Cells pretreated for 5 min with DNP, an oxidative phosphoryiation uncoupler, at concentrations reported to markedly disrupt mitochondrial function [19–21] showed no apparent reduction in [^{195}Au] cell association relative to control at any concentrations. Cells pretreated for 60 min with NaF, an inhibitor of phagocytosis and pinocytosis [22], also had no effect on [^{195}Au] cell association.

Effect of extracellular thiols on [^{195}Au]*AF and* [^{195}Au]*TEPG cellular association.* We previously reported [10] that the cellular association of AF is inhibited by preincubating the compound with both fetal calf serum and purified albumin. Like AF, increasing concentrations of FCS and albumin also inhibited cellular association of [^{195}Au]TEPG in a dose-dependent manner (data not shown). Inhibition of uptake was due, presumably, to the interaction of AF and TEPG gold with the free cysteine 34 sulfhydryl group of the albumin molecule [8, 23, 24].

Auranofin, TEPG and other gold I thiolate complexes also interact with other low molecular weight thiols such as cysteine, methionine and glutathione [8, 25, 26]. Panels A and B of Fig. 6 show the effect

Table 2. Effects of NEM, DNP, and NaF pretreatment on cellular association of [^{195}Au]auranofin and [^{195}Au]triethylphosphine gold chloride

Drug concn (μM)	Percent total cell-associated [^{195}Au] NEM AF	NEM TEPG	DNP AF	DNP TEPG	NaF AF	NaF TEPG
0	100	100	100	100	100	100
0.1	96 ± 7	93 ± 0.3	107 ± 5	97 ± 8		
1	94 ± 4	87 ± 5	98 ± 7	103 ± 9		
10	53 ± 5	74 ± 4	97 ± 8	98 ± 2	103 ± 0.6	99 ± 3
100	24 ± 3	23 ± 3	93 ± 12	98 ± 5	102 ± 4	89 ± 8
1,000	15 ± 2	17 ± 2			106 ± 7	93 ± 3
10,000					104 ± 1	83 ± 7

RAW 264.7 cells (1×10^6 cells/ml) were pretreated with DNP, NEM and NaF for 5, 30 and 60 min, respectively, and washed three times with PBS. The cells were then treated with [^{195}Au]-radiolabel (5 μM) for 10 min; the reaction was stopped, and radioactivity was determined as described in Materials and Methods. AF values are from Ref. 10.

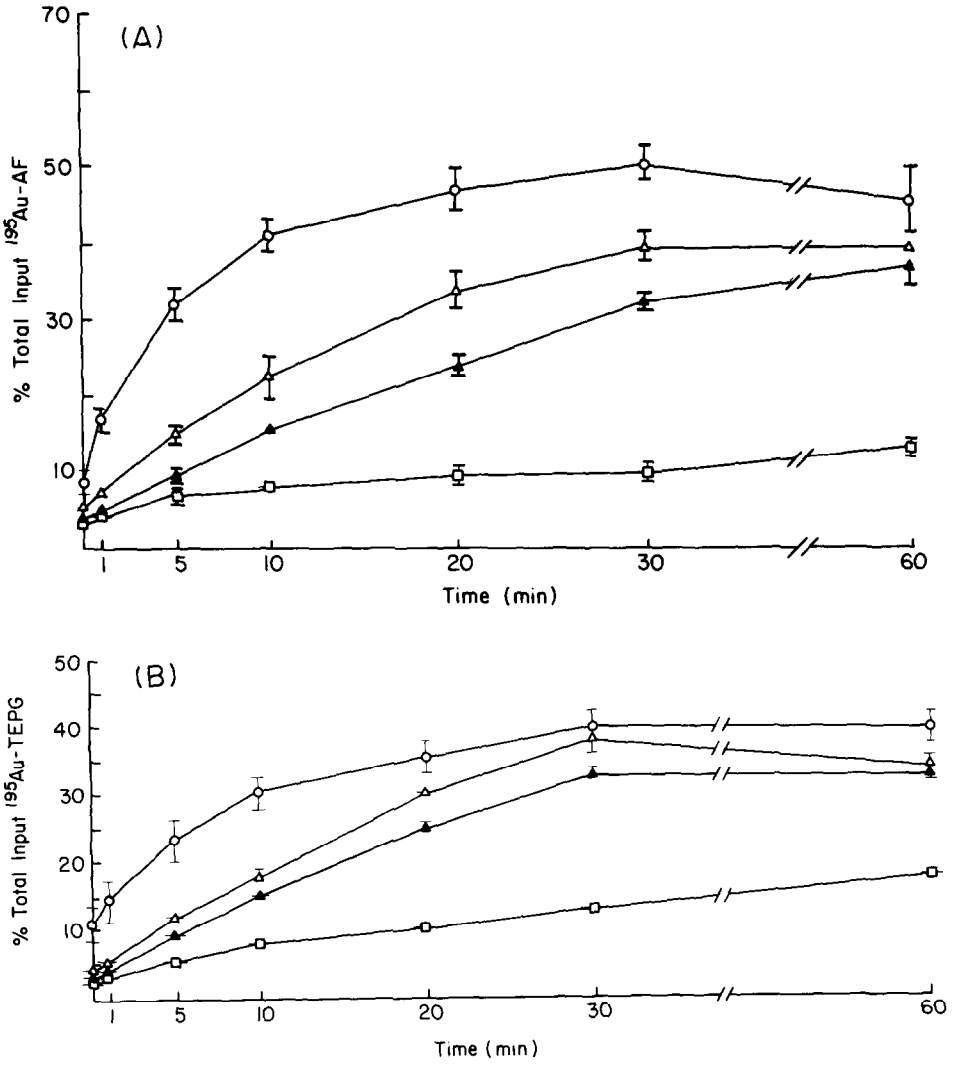

Fig. 6. Effects of GSH on the cell association of [^{195}Au]AF and [^{195}Au]TEPG. Experimental procedures are as described in Materials and Methods. Panel A represents data from experiments performed with 5 μM [^{195}Au]AF; panel B from those performed with 5 μM [^{195}Au]TEPG. The values shown are means and standard deviations from three separate experiments performed in triplicate. Key: (○—○) control; (△—△) 100 μM GSH; (▲—▲) 200 μM GSH; and (□—□) 500 μM GSH.

of preincubating 5 μM [^{195}Au]AF and [^{195}Au]TEPG with increasing concentrations of glutathione. Incubation of both compounds with GSH reduced cell association and uptake in a concentration-dependent fashion. The percent inhibition relative to control values was similar for AF and TEPG. Even at the highest concentration of GSH (500 μM), which represents a 100-fold excess of GSH to drug, 10% of AF-gold and 15% of the TEPG-gold still associated with cells after 60 min.

Dissociation of radiolabeled AF and TEPG. The dissociation of [^{195}Au] from cells preloaded with radiolabeled AF and TEPG was measured as described in Materials and Methods. In a previous paper [10], we demonstrated that the dissociation of radiolabeled AF is time and temperature dependent. As shown in Fig. 7, dissociation of [^{195}Au] from TEPG-loaded cells also increased as a function of incubation time in fresh medium. Dissociation was also temperature dependent. Essentially, no [^{195}Au] dissociated from cells at 4°, whereas at 37° [^{195}Au] derived from TEPG was observed in the incubation medium. This dissociation at 37° may be explained, in part, by the toxic effects of the drug which result in alterations in membrane integrity and function and cell lysis with subsequent release of gold from cells.

Panels A and B of Fig. 8 demonstrate the effect of incubating preloaded cells with increasing amounts of GSH in the efflux medium. Dissociation of [^{195}Au] increased for both compounds as the concentration of GSH increased. After 60 min of incubation with 1000 μM GSH, only 20–25% of the gold was retained by the cells, representing a 2- to 3-fold decrease in cell-associated gold relative to control. Our data are consistent with a report by

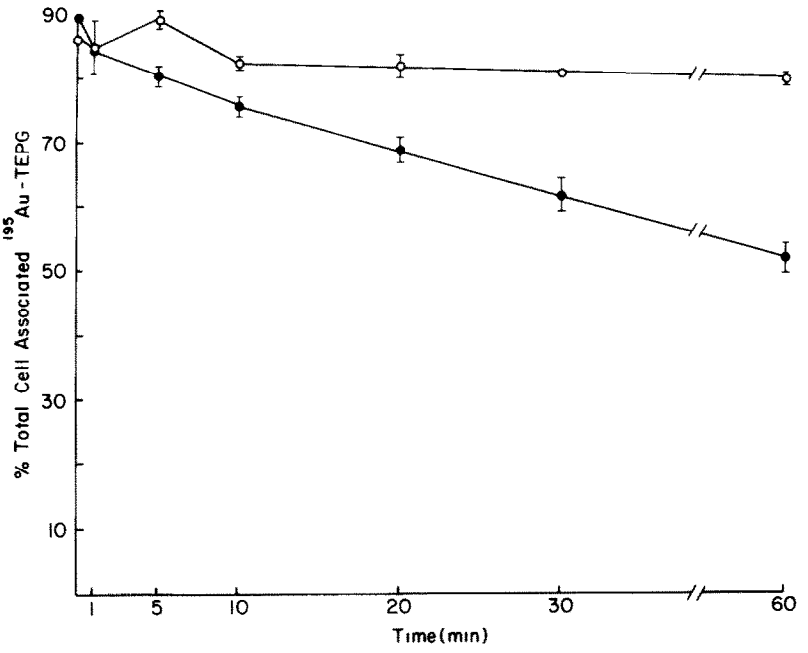

Fig. 7. Dissociation of cell-associated [^{195}Au] from radiolabeled TEPG. Experimental procedures are as described in Materials and Methods. The values shown are means and standard deviations from two separate experiments performed in triplicate. Key: (○—○) 4°; and (●—●) 37°.

Mirabelli *et al.* [27] who demonstrated that the addition of 2-mercaptoethanol to the medium of P388 leukemia cells preincubated with [^{195}Au]AF results in a decrease in the amount of cell-associated gold.

DISCUSSION

To further investigate the thiol exchange mechanism previously proposed [10], we have studied the cellular interactions of triethylphosphine gold chloride (TEPG), an alkylphosphine gold complex that differs from AF in that it possesses a chloride rather than TATG as a leaving group (Fig. 1). TEPG, like AF, is an orally active chrysotherapeutic agent [14, 28] that has similar effects in many experimental systems including inhibition of a number of inflammatory enzymes [29], suppression of lesions in adjuvant-induced arthritis [14], and inhibition of superoxide production by mouse peritoneal macrophages [9].

Since we believe that the sequential thiol exchange mechanism is a generic process, TEPG's cellular uptake, distribution, efflux and reactivity towards sulfhydryl reagents such as NEM, or GSH, should qualitatively, if not quantitatively, be similar to AF since the compounds are essentially the same (Au-Et_3P) after displacement of the leaving groups. Data presented in this and a previous paper [10] support the hypothesis that the rate-limiting step in cell association (Fig. 2A) is the substitution of membrane-localized sulfhydryl groups for the TATG of AF or chloride of TEPG. Thus, the temperature dependence for initial cell association derives from the energy required for this process. The rate of cell association of a gold-triethylphosphine containing compound should vary as a function of the lability of the leaving groups. Chloride is a better leaving group than TATG [17]. Consequently, we would predict that TEPG would associate with cells faster, and the energy of activation for that process would be lower, than that of AF.

Our data indeed demonstrate that cell association of TEPG, like AF, was concentration (Fig. 3), time (Fig. 4), and temperature dependent (Table 1) and, although the overall responses were qualitatively similar between the two compounds, some differences were observed. Figures 3 and 4 show that more gold from TEPG associated with cells. This increased cell association resulted, perhaps, from the greater leaving tendency of the chloride of TEPG which may facilitate interaction with thiols not seen with AF. The initial rates of cell association for TEPG and AF, measured over a range of temperatures, differed significantly (Table 1) and, generally, TEPG associated with cells at a greater rate than AF. The energy of activation as derived from the Arrhenius plot (Fig. 5) was lower for TEPG association than AF.

Data demonstrating that: (1) AF and TEPG cell association and uptake were inhibited by NEM (Table 2) and GSH (Fig. 6) in a concentration-dependent fashion, (2) intracellular distribution of gold from the two compounds were qualitatively similar (data not shown), and (3) dissociation of gold from AF and TEPG was increased to the same extent by the addition of extracellular GSH to the efflux incubation medium (Fig. 8) are consistent with the proposed mechanism in that each drug displayed the same behavior after displacement of the leaving

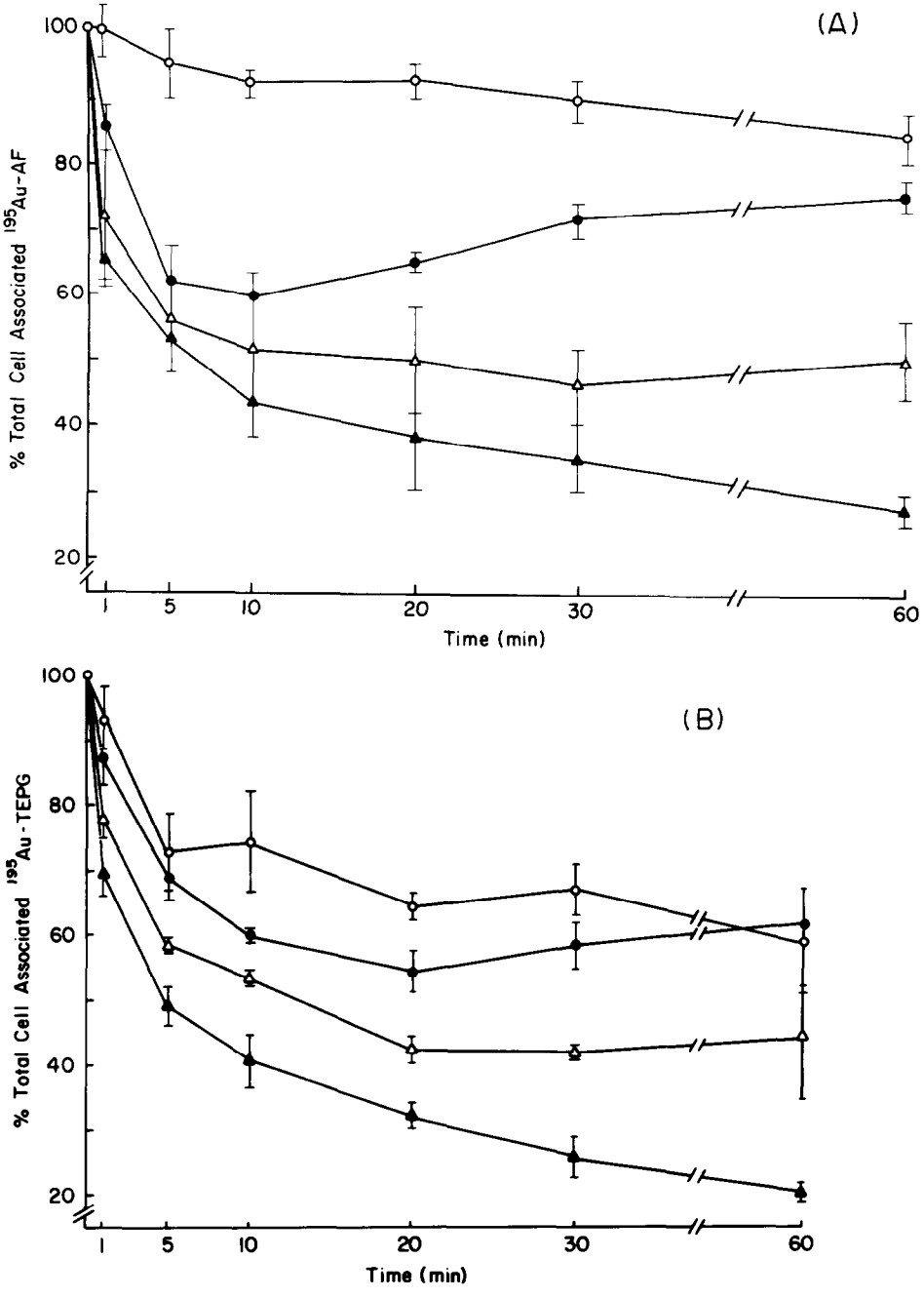

Fig. 8. Effects of GSH on dissociation of cell-associated [^{195}Au]-radiolabel. Panel A represents data from experiments performed with [^{195}Au]AF and panel B from those performed with [^{195}Au]TEPG. The values shown are means and standard deviations from two separate experiments performed in triplicate. Key: (○—○) control; (●—●) 250 μM GSH; (△—△) 500 μM GSH; and (▲—▲) 1000 μM GSH.

groups. In addition, the effect of extracellular GSH on uptake and efflux of Au from TEPG and AF, and the ineffectiveness of metabolic inhibitors on association, extend the concept that the interactions of these compounds depended solely on the reactivity of the gold and its ligands and the availability of cellular and membrane sulfhydryl groups. These and previous data further confirm that the active moiety of chrysotherapeutic agents is the gold and that ligands attached to the gold alter pharmacokinetics and intracellular distribution.

The generic nature of the ligand exchange model suggests that approaches to new chrysotherapeutics should focus on the effects of various ligands on the rates of various sulfhydryl exchange processes. Alterations in the sulfhydryl exchange potential may alter absorption, distribution and intracellular disposition of gold compounds. Introduction of ligands

with appropriate bulk or lipophilicity may, by stearic hindrance or other mechanisms, result in preferential interactions with selected cellular sulfhydryl-containing macromolecules.

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