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INHIBITION OF SEVERAL ENZYMES BY GOLD COMPOUNDS II. β-GLUCURONIDASE, ACID PHOSPHATASE AND L-MALATE DEHYDROGENASE BY SODIUM THIOMALATORAURATE (I), SODIUM THIOSULFATOAURATE (I) AND THIOGLUCOSOAURATE (I)

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Bovine liver β-D-glucuronide glucuronohydrolase, EC 3.2.1.32), wheat germ acid phosphatase (orthophosphoric monoesterphosphohydrolase, EC 3.1.3.2) and bovine liver L-malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37) were inhibited by a series of gold (I) complexes that have been used as anti-inflammatory drugs. Both sodium thiosulfatoaurate (I) (Na AuTs) and sodium thiomalatoraurate (NaAuTM) effectively inhibited all three enzymes, while thioglucosoaurate (I) (AuTG) only inhibited L-malate dehydrogenase. The equilibrium constants (K_I) ranged from nearly 4000 μM for the NaAuTM-β-glucuronidase interaction to 24 μM for the NaAuTS - β -glucuronidase interaction. The rate of covalent bond formation (k_p) ranged from 0.00032 min^{-1} for NaAuTM - β -glucuronidase formation to 1.7 min^{-1} for AuTG -L-malate dehydrogenase formation. The equilibrium data shows that the gold (I) drugs bind by several orders lower than the gold (III) compounds, suggesting a significantly stronger interaction between the more highly charged gold ion and the enzyme. Yet the rate of covalent bond formation depends as much on the structure of the active site as upon the lability of the gold-ligand bond. It was also observed that the more effective the gold inhibition the more toxic the compound.

KEY WORDS: Rheumatoid Arthritis, Gold (I) Drugs, Enzyme Models

INTRODUCTION

Although no drug has been found which allows complete remission of rheumatoid arthritis, and few which induce partial remission, nevertheless gold (I) drugs are among the few which offer the best chance based on clinical trials. Administration is often given under the name of chrysotherapy[1]. There is still no known mechanism as to how gold (I) reduces inflammation since there is no specific physiological function which is attributed to the gold ion; called a xenobiotic metal ion.

Gold (I) halides are unstable, disproportionating to gold (O) and gold (III). To stabilize it soft, class b ligands such as thiohalides, theiothers and phosphines are attached to the gold atom[2,4]. Thiol ligands have been chosen since serum thiols are evidently reduced during rheumatoid arthritis[5], and this enables the gold-ligand thiol to survive the transportation process to the site of inflammation. Much evidence suggests that the ligand can dissociate soon after administration[6,7], and there is some

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evidence to suggest that the exogenous ligand may be partly responsible for some biological effects, as observed from the studies on penicillamine[8].

Shaw has proposed a mechanism[9] in which the ligand is replaced by a thiol active group.

$$RSH + Au\text{–}L \rightarrow R\text{–}S\text{–}AuL + H^+ \quad (1)$$

$$R\text{–}S\text{–}AuL + RSH \rightarrow RS\text{–}Au\text{–}SR \quad (2)$$

This may be the method of transportation of the gold (I) to the inflammed site, but the inhibition of the hydrolytic enzymes or other molecules may necessitate groups other then the sulfur atom to stabilize the gold (I) atom. To complicate matters, the gold complexes (eg., gold (I) thiomalate) exist as polymers in aqeuous solution [9,10], and they probably exist that way *in vivo*.

The gold compounds that were chosen for this investigation, sodium gold (I) thiomalate (NaAuTM) (11), sodium gold (I) thiosulfate (NaAuTS)[12,13] and gold (I) thioglucose (AuTG)[14,15] all have been shown to have theraputic value. These three complexes tended to concentrate in the liver, spleen and kidney of rats and they were primarily eliminated by urinary excretion[16]. *In vivo* uptake of NaAuTM by liver lysosomes was demonstrated by Davies *et. al.*[17].

We intend to present inhibition constants in a manner similar to that which was undertaken using the gold (III) complexes (see preceding paper) and compare the binding effectiveness of gold (I) complexes with gold (III) compounds. We will also investigate the effect of the free ligands on the enzyme activity.

EXPERIMENTAL

Materials

Nicotinamide adenine dinucleotide (NAD), sodium thiomalate, thioglucose and sodium thiosulfate were purchased from Sigma. NaAuTM, NaAuTS and AuTG were purchased from K and K laboratories. Bovine liver L-malate dehydrogenase, also purchased from Sigma, was further purified by elution with 0.10 M phosphate buffer, pH 7.0 through a 2.0 × 50 cm column of Sephadex G-100. Gel electrophoresis of this material produced only one major band, and no further purification was carried out. All other material was reported in the preceding paper or was of the highest grade possible. The assay for L-malate dehydrogenase was carried out as previously described[18]. All other assay procedures were explained in the gold (III) studies.

Enzyme Studies

The kinetic and equilibrium methods have been described in the preceding paper. It was observed after a number of experiments that aquation of the gold (I) complexes did not produce significant increase or decrease in the inhibition upon comparison with the non-aquated compounds. Therefore, the time factor for aquation was neglected in most studies. The L-malate dehydrogenase studies were run at pH 7.0 and 25°C.

Various concentrations of free ligands, sodium thiomalate, sodium thiosulfate and thioglucose were added to the enzymes in the presence or absence of their respective

gold complexes. The ligand-enzyme or ligand-gold complex-enzyme mixtures were allowed to mix for 24 h prior to assay.

Several of the enzymes were allowed to be partially or completely inhibited by various concentrations of gold complexes for 24 h. The mixtures were then either diluted with a 0.10 M phosphate buffer, pH 7.0, and passed through a 2.0 × 35 cm column containing Sephadex G-100, or they were dialyzed against the same phosphate buffer for several hours prior to assay. Lastly, some of these inhibited complexes were mixed with various concentrations of cysteine and allowed to stand for a period of several hours. Each of these mixtures was tested for regenerated enzyme activity.

RESULTS

β-Glucuronidase

The inhibition of bovine liver β-glucuronidase by various concentrations of NaAuTM was carried out over a period of 40 h during which less than 5% of the control's activity was lost on standing. The curves followed first order kinetics, and the log of the % remaining activity verses time is presented in Figure 1. The plot of the

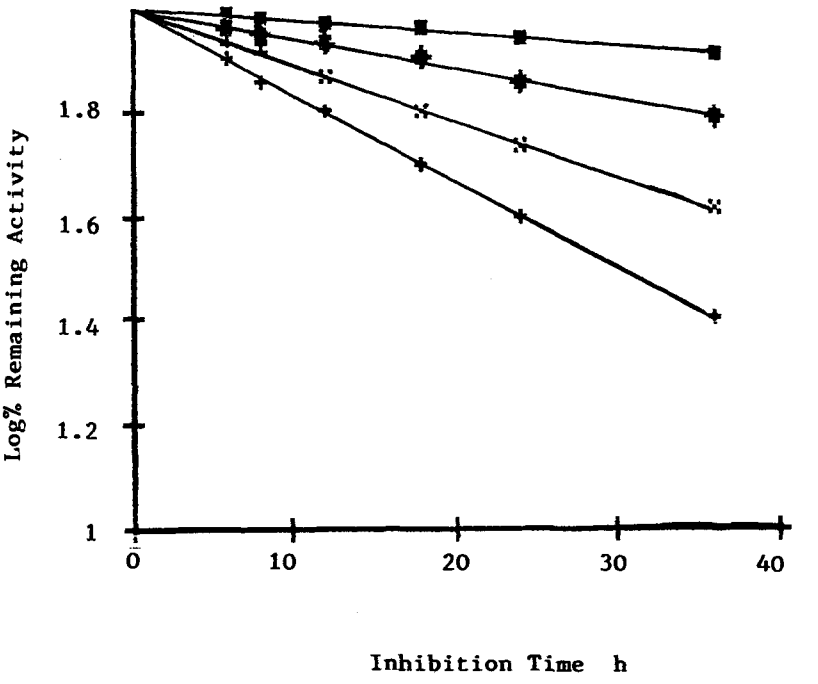

FIGURE 1 Inhibition of β-Glucuronidase by NaAuTM. The log % remaining activity is plotted against NaAuTM concentration. The enzyme concentration is maintained at 30 nM, and the inhibition reactions were carried out in a 0.1 M phosphate buffer at pH 7.0 and 25°C, while the assay was run in a 0.1M, pH 5.0 acetate buffer. The NaAuTM concentrations were; -■-2.0 mM, -✦-4.0 mM, -✕-6.0 mM and -+-8.0 mM. The control, not shown, exhibited no decrease in activity over the 40 h measuring period.

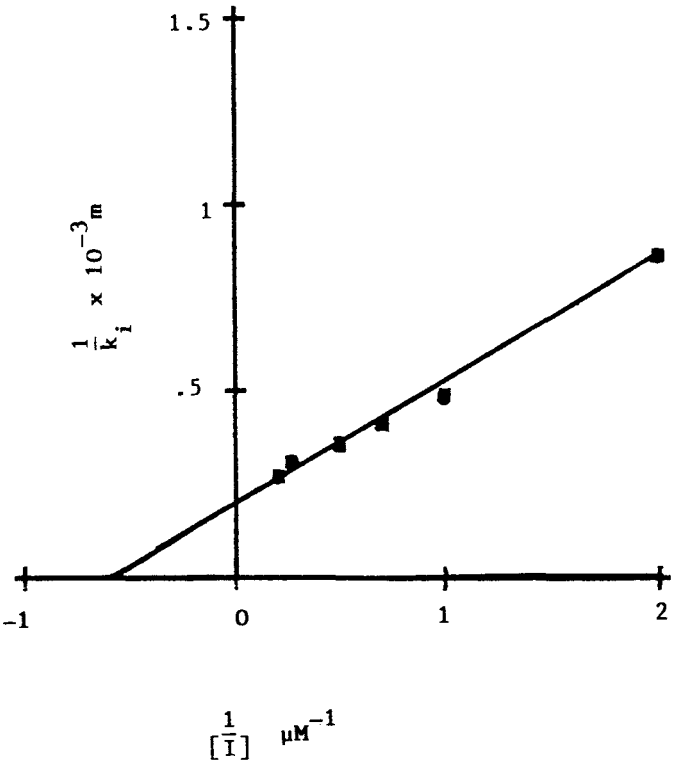

FIGURE 2 Double Reciprocal Plot for the Rate of Inhibition by NaAuTM of β-Glucuronidase Versus NaAuTM Concentration. The rates were calculated from the slopes in Figure 1.

reciprocal of the pseudo first-order rate constants verses the reciprocal of the NaAuTM inhibitor concentrations is shown in Figure 2. The values of k_P (I/K_i intercept) for the irreversible gold complex-enzyme formation and K_I ($1/[I]$ intercept) are given in Table III. The K_I and k_P values, respectively, represent the reversible equilibrium constant for gold complex-enzyme association and the rate of formation of the irreversible gold-enzyme adduct as illustrated in the preceding paper. A similar analysis was performed using NaAuTS as the enzyme inhibitor. The inhibition was carried out over a period of 20 h using various gold complex concentrations and the log plots are presented in Figure 3. The double reciprocal plot, similar to that presented for NaAuTM, is shown in Figure 4, and the K_I and k_P values obtained from the slopes in Figure 4 are presented in Table III.

Experiments in which lower gold complexes were allowed to interact with β-glucuronidase were run for longer periods of time until the inhibition reached equilibrium. The activity was read after 20 h, and a Lineweaver-Burk double reciprocal plot was prepared. The results for the NaAuTM studies are illustrated in Figure 5, and the K_I value calculated from the slopes in this experiment is given in Table III. It appears from the interaction that this gold compound is acting as a purely competitive inhibitor. A similar type of experiment (not shown) was attempted using NaAuTS as

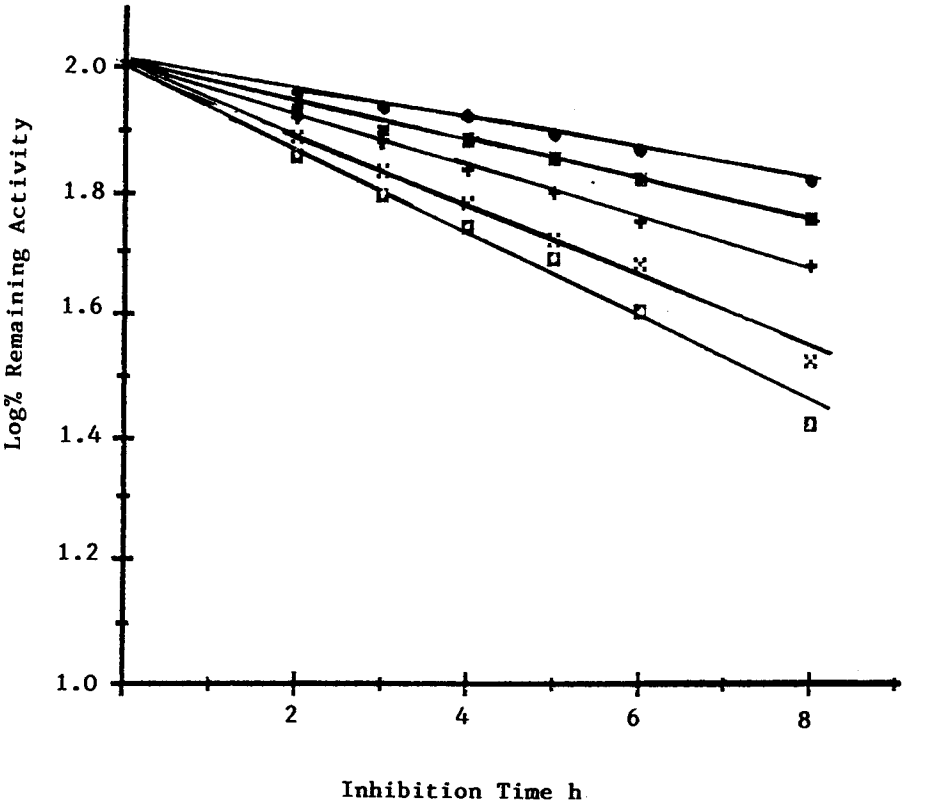

FIGURE 3 Inhibition of β-Glucuronidase bn NaAuTS. The log remaining activity is plotted against NaAuTS concentration. See Figure 1 for the experimental conditions. The NaAuTS concentrations were; -●-30 mM, -■-. 40 mM, -+-50 mM, -※-60 mM and -□-80 mM.

the inhibitor. Yet the data points generated from this study were quite erratic and there was no common intersecting point, suggesting that an equilibrium state was not reached. However, an average K_I value was computed from these lines and is presented in Table III.

When the free sodium thioalate ligand was added to the enzyme solution in the absence of the gold complex there was virtually no loss of the original activity (Table I). The free ligand concentrations were as much as five times greater than the gold complex concentrations. The enzyme activity was then measured in the presence of both the gold complex and the free ligand, but no protection from or enhancement of the enzyme inhibition by the free ligand was observed. A similar set of experiments were performed using the free sodium thiosulfate ligand in the presence and absence of its gold atom (Table I). In these experiments there did appear to be a slight protection of the enzyme from the gold compound by the thiosulfate ligand.

β-glucuronidase was subjected to gel filtration on Sephadex G-100 after inhibition by various concentrations of both NaAuTM and NaAuTS, and no significant increase in activity was observed. The enzyme was also inhibited by large excesses of gold complex for about 6 h, or until no activity was observed. Subsequent dialysis of

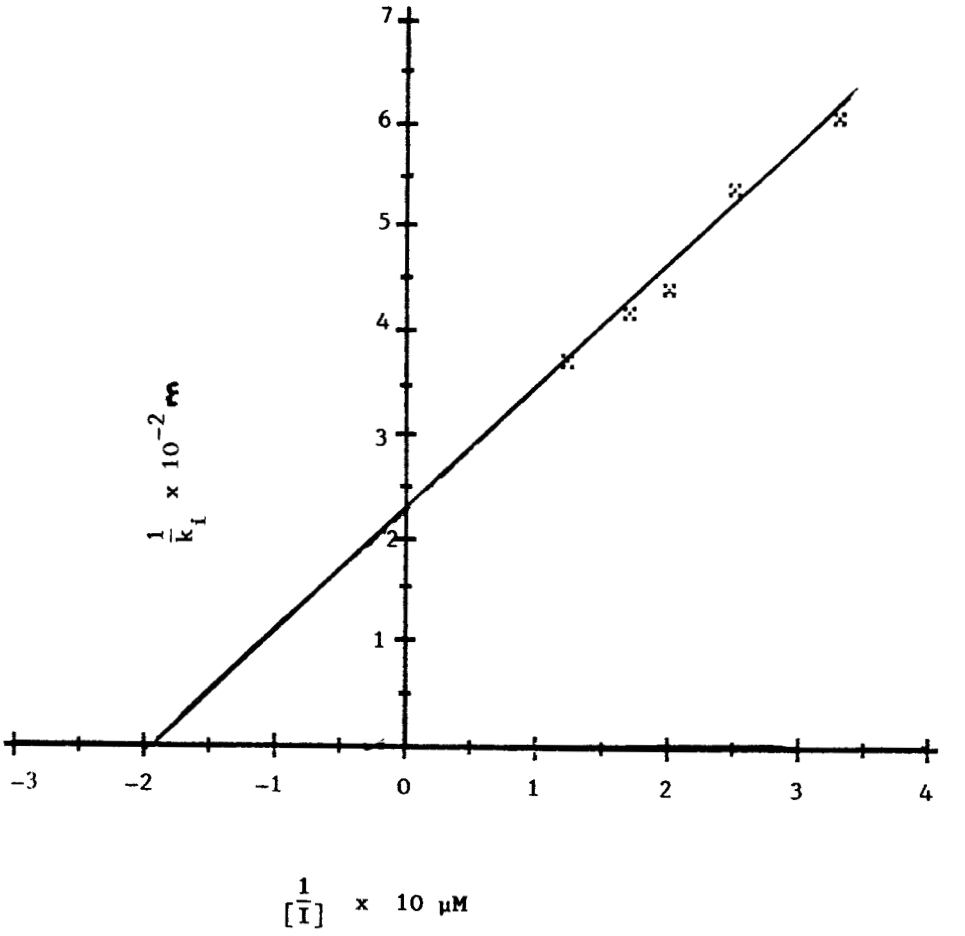

FIGURE 4 Double Reciprocal Plot for the Rate of Inhibition of NaAuTS of β-Glucuronidase Versus NaAuTS Concentration. The rates were calculated from the slopes in Figure 3.

the inhibited enzyme for 6 to 12 h in order to remove the excess gold complex did not regenerate any activity. Lastly, an attempt was made to reactivate the inhibited enzyme by using a large excess of cysteine, which is known to complex strongly with heavy metals, and no regeneration of activity took place under these conditions.

Acid Phosphatase

Kinetic studies of the type performed on β-glucuronidase, were attempted using the gold complexes with wheat germ acid phosphatase, however, the data did not generate reproducible linear curves. Thus, only 24 h inhibition studies at lower gold complex concentration were carried out. Double reciprocal plots were constructed using both NaAuTM and NaAuTS, as shown in Figures 6 and 7. The k_i values were determined

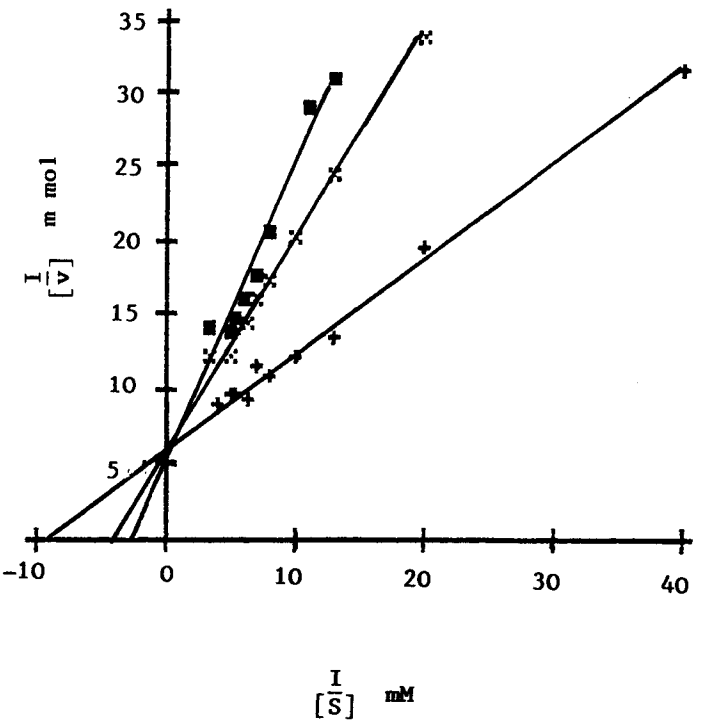

FIGURE 5 Lineweaver-Burk Double Reciprocal Plot of the Activities of the 24 h NaAuTM Inhibited β-Glucuronidase using Phenolphthaleinglucuronate as Substrate. The reaction conditions are given in Figure 1. The concentrations of NaAuTM inhibitor were; -×-1.0 mM and -■-2.0 mM. -+-represented the uninhibited enzyme.

from the slopes, and the values are presented in Table III. A mixed-inhibition type was produced by NaAuTM, while a purely competitive type was observed for NaAuTS.

Addition of both the free thiomalate or thiosulfate ligands to the enzyme in the presence or absence of the gold compounds yielded the results shown in Table II. The concentration of ligand used in these experiments was approximately 10 to 20 times in excess of the gold compound. Only a negligeable amount of activity was lost by mixing the free ligand with the enzyme, however, when the free lignad and gold complex were mixed with the enzyme the inhibition was reduced by 25 to 50%.

L-Malate Dehydrogenase

Kinetic inhibition experiments were carried out with porcine heart mitochondrial L-malate dehydrogenase in a manner similar to those with the other two enzymes. Various concentrations of NaAuTM, NaAuTS and AuTG were used, and the first order rate curves were constructed from the time-dependent inhibition studies. The double reciprocal plots for the first order rate constants were plotted versus the inhibitor concentrations, and the results are presented in Figure 8. The reversible

TABLE I

Effect of Free Ligands and Gold Complexes on the Activity of β-Glucuronidase When Mixed Separately and Together. The mixtures were allowed to stand for 24 h in a 0.1 M phosphate buffer, pH 7.0 at 25°C before activity measurements were made. [Enzyme] = 30 nM.

[Ligand] mM NaTM	[Au-Compound] mM NaAuTM	Activity μmol/m
0	0	3.52
10	0	3.54
0	4.0	2.65
10	4.0	2.64
40	0	3.50
0	8.0	2.18
40	8.0	2.25
NaTS	NaAuTS	
0	0	3.65
0.5	0	3.60
0	0.02	1.97
0.5	0.02	2.07
2.0	0	3.57
0	0.04	1.55
2.0	0.04	1.67

All tests were performed in triplicate, with an error of no greater than ± 5%.

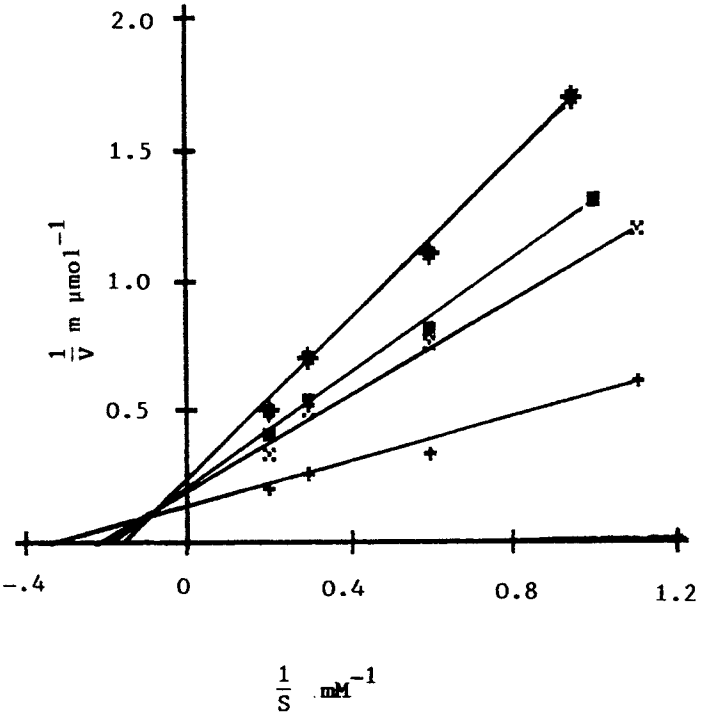

FIGURE 6 Double Reciprocal Plot of the Activity of the 24 h NaAuTM Inhibited Acid Phosphatase using O-Carboxyphenylphosphate Substrate. The inhibition was carried out in a 50 mM tris pH 7.0 buffer at 25°C, while the assay was run in a 0.1 M acetate pH 5.0 buffer. The concentrations of NaAuTM inhibitor were; -✦-410 mM, -■-270 mM and - ✕ -200 mM. - + - represented the unihibited enzyme.

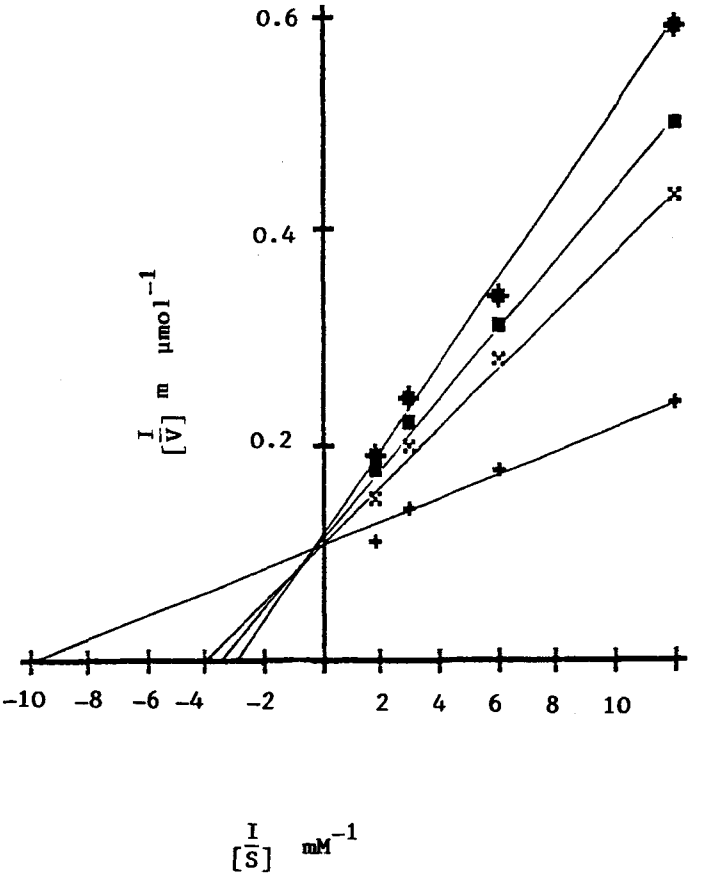

FIGURE 7 Lineweaver-Burk Double Reciprocal Plot of the Activity of the 24 h NaAuTS Inhibited Acid Phosphatase Versus O-Carboxyphenylphosphate Substrate Concentration. The experimental conditions are given in Figure 6. The concentrations of NaAuTS were; -✦-270 mM, -■-200 mM and -✕-140 mM. -+-represented the inhibited enzyme.

binding constant, K_I, and the irreversible rate constant, k_p, were determined for each of the inhibitors, and the results are given in Table IV.

DISCUSSION

The inhibitory effect of the gold drug NaAuTM was tested against β-glucuronidase, acid phosphatase and L-malate dehydrogenase. Rate studies using high concentrations and 24 h equilibrium inhibition using lower concentrations of drug were performed with this enzyme. The association (K_I) between the enzyme and the NaAuTM complex was 100 times weaker than for the gold (III) halo compounds as previously reported, and the rate (k_p) of gold-enzyme covalent bond formation for the gold thiomalate complex was 16 times slower than that for the halo compound. The weak

TABLE II
Effect of Free and Gold Complexes on the Activity of Acid phosphatase. The mixture was allowed to stand for 24 h before activity measurements were taken. [Enzyme] = 25 nM.

[Ligand] mM NaTM	[Au-Complex] mM NaAuTM	Activity μmol/min
0	0	5.90
3	0	5.90
0	0.26	3.00
3	0.26	4.30
0	0.40	2.57
0	0.40	3.05
NaTS	NaAuTS	
0	0	8.80
2.3	0	8.60
0	0.13	6.80
2.3	0.13	7.70
0	0.26	5.92
2.3	0.26	6.72

All tests were run in triplicate, with error of no greater than ± 5%.

TABLE III
Compilation of the Rate Constants, k_p, and Equilibrium Constants, K_I, from the Rate Studies, and the Equilibrium Constants, K_I, from the Equilibrium Studies.

Enzyme	Gold Complex	Rate* $k_p \times 10^2 min^{-1}$	Rate* $K_I \mu M$	Equilibrium* $K_I \mu M$
β-Glucuronidase	NaAuTM	0.032	3940	3980 ± 350 (C)**
	NaAuTS	0.45	52	24 ± 10
Acid Phosphatase	NaAuTM			190 ± 30 (M)**
	NaAuTS			105 ± 15 (C)**
Malate Dehydrogenase	NaAuTM	115	150	
	NaAuTS	130	91	
	AuTG	170	2600	

*An error no greater than ± 5% in rate or equilibrium constants.
**C is competitive inhibition and M is mixed inhibition.

enzyme-NaAuTM association reflects the stability of the ligand-gold bond. The slower rate is probably due to the greater difficulty in orienting the thiomalate ligand into the binding site of the enzyme, and the greater stability of the gold (I)-sulfur bond as compared to the gold (III)-halo bond, as previously suggested. The K_I value obtained from the equilibrium inhibition data is nearly identical to the value from the rate experiments. This suggests that inhibition of β-glucuronidase is achieved during the association of the gold complex with the enzyme and covalent bond formation merely stabilizes it.

Rate experiments for the inhibition of acid phosphatase by NaAuTM were attempted, but due to lack of reproducible data, generation of accurate curves was not possible. However, the 24 h inhibition curves using lower concentrations were reproducible and the calculated equilibrium constants show a 20 fold increase over those obtained in the β-glucuronidase experiments illustrating a more tightly bonded

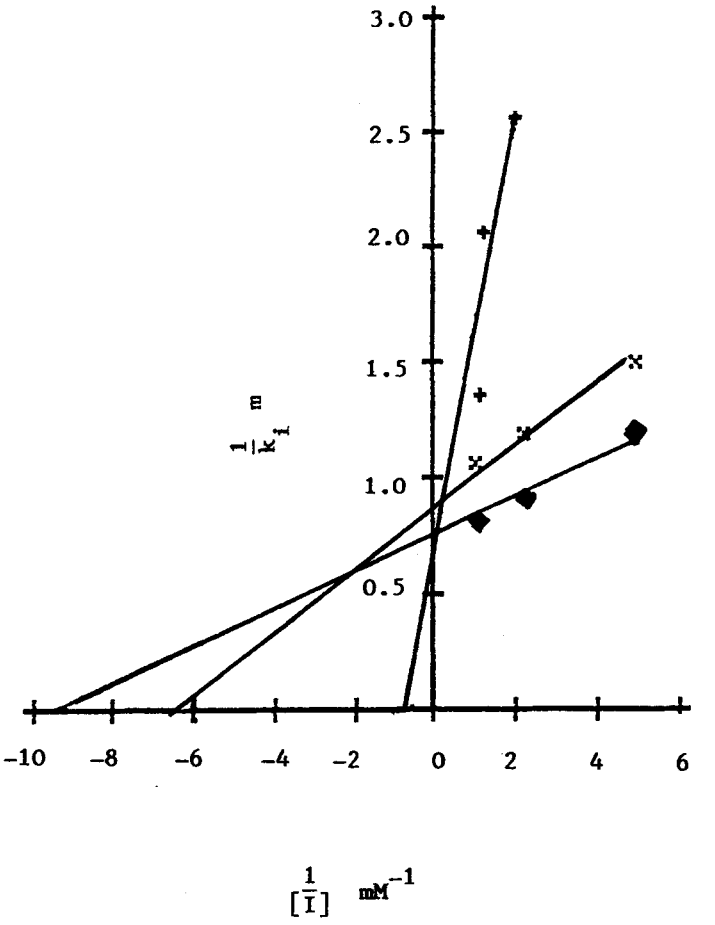

FIGURE 8 Double Reciprocal Plot of the Rate of Inhibition of L-Malate. Dehydrogenase Versus the Concentration of Several Inhibitors. The inhibition was carried out in a 0.1 M phosphate buffer pH 7.0 at 25°C, while the assay was run in a 0.1 M acetate pH 5.0 buffer. The inhibitors were; −◆−NaAuTS, −×−NaAuTM and −+−AuTG

TABLE IV
Toxicity Data for Gold Complexes in Mice.

Complex	MD* (mg Au/kg body weight)
$NaAuCl_4$	41
NaAuTS	37
NaAuTM	202
AuTG	825

*minimum lethal dose

adduct generated from a more easily displaceable ligand. The K_I is approximately 100 times weaker that the gold (III) value found in the previous work, thus following the same pattern with β-glucuronidase. The β-glucuronidase produced a competitive type inhibition while the acid phosphatase gave a mixed inhibition suggesting slight differences in the manner in which the enzymes bind the drug.

The effect of NaAuTM towards L-malate dehydrogenase was of interest since the ligand is similar to the substrate of the enzyme. It is observed that the K_I value and thus the formation of the gold—enzyme bond is similar to that for the acid phosphatase, but significantly stronger than that for the β-glucuronidase, suggesting a more stable adduct. Also, the rate of covalent bond formation is significantly greater than found for β-glucuronidase suggesting a more favorable orientation. The bond forms at the same rate as that for the gold (III) compounds with acid phosphatase from the previous study. Since L-malate dehydrogenase has an active sulfhydryl group it is highly likely that once the gold complex is correctly oriented the sulfur atom binds directly to the gold atom forming a stable thiol-gold adduct. The similarity in the equilibrium inhibition and rates of covalent bond formation for acid phosphatase suggests to us that there might be an active thiol group in one of the enzyme species as proposed by previous workers[19,20]. Addition of the free thiomalate ligand in the presence or absence of gold complex displayed no effect on the activity or inhibition of β-glucuronidase and only a slight effect on the inhibition of acid phosphatase. This supports the contention that the gold is the primary inhibition agent, and that the released ligand displays little or no effect. The final inhibited complex was quite stable since gel-filtration or cysteine addition failed to regenerate any activity.

A similar series of experiments on the three enzymes were performed using NaAuTS as the inhibitor. The K_I values for β-glucuronidase, L-malate dehydrogenase and acid phosphatase were all of the same magnitude, and lower than the corresponding NaAuTM-enzyme values. The largest differences occurred in the inhibition of β-glucuronidase in which the K_I value was approximately 170 times greater, and the k_p was 13 times greater than the corresponding NaAuTM values. The inability to get reproducible equilibrium data upon comparing the K_I values from rate and equilibrium experiments coupled with the larger rate constant suggests a faster reaction occurs between β-glucuronidase and NaAuTS, with the association stage not able to reach equilibrium prior to significant covalent bond formation. The gold thiosulfate compound binds more tightly to all three enzymes so this particular compound appears to be more reactive and less selective than the gold thiomlate compound. Once again the k_p for the L-malate dehydrogenese gold thiosulfate bond formation is significantly greater than that found for β-glucuronidase suggesting the involvement of the thiol group in the dehydrogenase molecule. No siginficant reversal of inhibition via cysteine complexation, gel filtration or blocking by excess free ligand was observed indicating that the binding is strong, terminally irreversible and not blocked by any dissociated ligand.

The only effective studies using AuTG was made by employing L-malate dehydrogenase since the other two enzymes were unable to be inhibited at a saturating gold (*I*) concentration. The very large K_I is due to bulkiness of the gold compound as well as its neutral charge. The importance of the neutral change may be reflected in the fact that the other ligands are highly charged at pH 7.0, and they are effective in eliciting inhibition towards all three enzymes. The k_p value is comparabhle to those of the other gold ligands suggesting a similarity with the other drugs in the enzyme-gold (I) covalent bond formation.

These gold (I) complexes have also been shown to have toxic effects[21]. Several compounds, listed in order of their toxicity, are presented in Table IV. It appears, based upon our results, that those complexes which have the strongest affinity for the target enzymes also produce the highest level of toxicity. The degree of effectiveness of any drug is its maximal theraputic effect versus the minmum toxic effect, and although some drugs may be very effective therapeutically they may also have a high degree of toxicity. The Au (I) complexes used in this study all have high therapeutic values depending on the type of therapy in which they are employed, while the Au (III) complexes are not used at all due to their great reactivity. It is difficult to predict the overall effectiveness of any potential drug from its structure, yet at the same time there are some characteristics that might be used in their preparation, such as the charge on the ligand and the strength of the bond attachment to the gold atom.

In conclusion, the enzymes used here are effective models for eliciting reactions towards the gold drugs. Both rate and equilibrium constants were generated which reflect the quantitative manner in which the drugs interact. As more data is gathered a clearer picture will develop concerning the therapeutic use of these compounds.

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