J. Enzyme Inhibition, 1989, Vol. 3, pp. 23–33
Reprints available directly from the publisher
Photocopying permitted by license only

INHIBITION OF HYDROLYTIC ENZYMES BY GOLD COMPOUNDS I. β-GLUCURONIDASE AND ACID PHOSPHATASE BY SODIUM TETRACHLOROAURATE (III) AND POTASSIUM TETRABROMOAURATE (III)

MING-TING LEE*, TAMANA AHMED and MICHAEL E. FRIEDMAN,**

Department of Chemistry, Auburn University, AL 36849–5312

(Received 28 September, 1988)

Purified bovine liver β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.32) and wheat germ acid phosphatase (orthophosphoric monoesterphosphohydrolase, EC 3.1.3.2) were inhibited with freshly dissolved and 24 h aquated tetrahaloaurate (III) compounds. Rate and equilibrium inhibition constants were measured. From this data two acid phosphatases species were observed.

Equilibrium inhibition constants ranged from 1 to 12.5 μM for the various gold compounds toward both enzymes. The first order rate constants ranged between 0.005 and 0.04 $min.^{-1}$ for most reactions with the exception of the fast reacting acid phosphatase which had values as high as 2.6 and 2.8 $min.^{-1}$

It is observed that the β-glucuronidase is rapidly inhibited during the equilibrium phase before the more slower reaction covalent bond formation takes place. The acid phosphatases form the covalent bonds more rapidly, especially the faster reacting species suggesting a unique difference in the active site geometry to that of the more slowly reacting species. The tightly bonded gold (III)-enzyme complex is probably the reason for its toxicity and non-anti-inflammatory use as a drug.

KEY WORDS: Rheumatoid Arthritis, Enzyme Models, Gold (III) Compounds.

INTRODUCTION

Lysosomal enzymes are considered to be important molecules in the pathogenesis of inflammatory diseases such as rheumatoid arthritis.[1,2] Included among these enzymes are β-glucuronidase and acid phosphatase.[3] During inflammation, these enzymes[4,5] are released into the synovial fluid and catalyze substantial hydrolytic breakdown of adjoining tissue.

Gold (I) complexes have been used for the mediation of various symptoms as well as a partial cure for rheumatoid arthritis for over half a century[6,7] while gold (III) compounds have yet to be found therapeutically useful. Although the therapeutic value of gold (I) complexes has been a subject of discussion,[8–10] a number of controlled studies have led to the conclusion that they have beneficial effects.[10–12] Several excellent review articles[13–15] covering the history and present activity of anti-inflammatory gold complexes, can be found in the literature.

A number of mechanisms have been suggested for the effect of these gold complexes on the inflammation process: the actions on prostaglandin synthesis,[16–18] the binding to collagen[19–21] the effect on the immune system[22–24] and the inactivation of lysosomal,[25–27] granulocytic[28,29] and other enzymes.[30–33] Investigations into these mechan-

* Present address Rosewell Park, Buffalo New York.
** Correspondence

isms, especially those involving protein binding and enzyme inactivation, have led workers[31,34] to suggest that gold (I) complexes preferentially bind to sulfhydryl or methionyl groups on the cell surfaces in accessible crevices. Evidence has been presented to suggest that gold (III) ions may also bind to proteins via sulfhydryl and methionyl [35] as well as through nitrogen bases such as those on lysyl and arginyl residues.[14] However, it has also been reported that gold (III) ions can also cause irreversible denaturation of some proteins via the oxidation of the thioester side chain of methionine to a sulfone and the sulfhydryl side chain of cysteine to a sulfoxide or disulfide.[15]

The first of our two studies were designed to investigate the extent of inhibition of some gold compounds, sodium tetrachloroaurate (III) and potassium tetrabromoaurate (III), to two representative and easily obtainable sources of enzymes; bovine liver β-glucuronidase and wheat germ acid phosphatase. The object was to provide kinetic and equilibrium parameters which will later be used as a means of determining the efficacy of gold drug binding to enzymes. Previous work in our laboratory[35] produced an effective inhibitory response to malate dehydrogenase and fumarase, which have been reported to have catalytically active side chains containing a sulfur atom. However β-glucuronidase has no cysteinyl or methionyl side chains,[36,37] while acid phosphatase may have a cysteinyl group near the active site.[38] It was of interest to determine how effectively they could be inhibited by the gold (III) halides.

It has been shown that many active site directed inhibitors first associate with the enzyme in a reversible manner, and then form a stable covalent or a coordinate covalent bond[38,39] as illustrated by equation 1.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_p} E\text{–}P + X \qquad (1)$$

k_{-1}/k_1 is the reversible dissociation constant (K_I) of the enzyme-gold halide compound $E \cdot I$, and k_p is the rate constant for the formation of the covalent enzyme-gold compound (E–P). X represents any displaced ligand from the gold compound. The rate constant of inhibition, k_i, can be calculated from a first order plot of the enzyme inhibition by the gold compound, and then by graphing the inhibition concentration verses the reciprocal of the rate constant, the first order rate constant, k_p, and the reversible inhibitor, K_I, can be obtained by using equation 2.

$$1/k_i = (K_I/k_p)/[I] + 1/k_p \qquad (2)$$

EXPERIMENTAL

Chemicals

Fresh beef liver from which the β-glucuronidase was prepared was obtained from the Auburn University poultry science department. Beef liver β-glucuronidase, wheat germ acid phosphatase, bovine serum albumin and phenolphthaleinglucuronic acid were purchased from Sigma. Sodium tetrachloraurate (III) and potassium tetrabromoaurate (III) were a gift from Michael Cleare of the Johnson-Matthey Co., Reading, England. CM-cellulose, DEAE-cellulose and tris buffer were also purchased from Sigma, while Sephadex G-50 and G-200 were obtained from Pharmacia. Purified ammonium sulfate, glycine, acrylamide, N,N-methylenebisacrylamide, N,N,N′,N′-

tetramethylethylenediamine (TEMED), and *o*-carboxyphenylphosphate were purchased from Eastman. The Folin-Ciocalteu reagent was prepared in our laboratory. All other chemicals were of the best grade commercially available.

Enzyme Purification and Assay

The method of Bernfeld *et al.*[41] was employed for the purification of the liver β-glucuronidase with only slight modification. Ammonium sulfate fractionaction, G-200 Sephadex, DEAE-Sephadex and CM-cellulose fractionation were employed. Gel electrophorsis was used to determine the purity. The specific activity was found to be 83 μmol/m mg using phenolphthaleinglucuronate as the substrate.

The Sigma wheat germ acid phosphatase was purified on a 2.0 × 115 cm Sephadex G-50 column by eluting with a 50.0 μmM tris buffer at pH 8.0. The specific activity was 6060 μmol/min/mg using *o*-carboxyphenylphosphate as the substrate.

The activity of β-glucuronidase was measured using the method of Talalay *et al.*[42] as modified by Fishman *et al.*[43] The reaction was carried out in acetate buffer at pH 5.0 and 25°C and the color developed at pH 10.8 at 37°C. The absorbance was measured at 540 nm on a Gilford Model 250 Recording Spectrophotometer.

Acid phosphatase was assayed according to the method of Brandenberger and Hanson[44] as modified by Hofstee.[45] The absorbance was measured at 300 nm.

The activities of both enzymes were converted from absorbance to μmol/min. units by employing standard curves of phenolphthalein for β-glucuronidase and salicyclic acid for acid phosphatase.

The protein concentration was determined according to the method of Lowry *et al.*[46] Crystallized bovine serum albumin was employed as the standard.

Gel-Electrophoresis

Gel electrophoresis was carried out according to the procedure of Laemmli and Maizel.[47] Separating gels of 7.5% and 15% were prepared for the β-glucuronidase and acid phosphatase studies, respectively. Identification of the protein bands were determined by Coomassie blue staining. Location of β-glucuronidase was also determined by emersing the gel in a 0.1 mM phenolphthaleinglucuronide substrate solution for 30 min. after completion of electrophoresis. The red phenolphthalein band was developed in a 0.10 M glycine buffer.

Enzyme Inhibition

Stock solutions of $NaAuCl_4$ and $KAuBr_4$ were prepared in the appropriate buffer for all studies, and concentrations from 5 to 25 M were employed. β-Glucuronidase studies were carried out in 0.1 M, pH 7.0 phosphate solution while those employing acid phosphatase were in a 50 mM, pH 7.0 tris solution.

The stock gold solutions were freshly prepared for the non-aquated studies, and rapid transfer, from 5 to 10 s after dissolution of the solid, were carried out to minimize aquation. The final concentrations of gold halide were 0.5 to 10.0 μM, while the enzyme concentrations were held constant at 30 nM for β-glucuronidase and 25 nM for acid phosphatase. All enzyme reactions were run at 25°C. Ten to fifty ml aliquots were periodically removed for assay.

A similar procedure was carried out in the aquation studies with the exception that

the gold halide solutions were allowed to aquate in the appropriate buffer for 24 h prior to being transferred to the enzyme solutions.

Equilibrium studies were carried out by mixing lower concentrations of gold halide and allowing the reaction to stand until the activity remained constant.

RESULTS

Inhibition of β-Glucuronidase

The rates of inhibition of β-glucuronidase by freshly prepared and 24 h aquated $NaAuCl_4$ were measured first. These complexes produced hyperbolic decreases in activity over a range of concentrations from 0.5 to 4.0 μM. The inhibition determined over a 24 h period by the non-aquated samples, was slightly more rapid than that produced by the aquated samples. Only a small decrease took place after 24 h, and longer time studies were omitted due to significant losses in enzyme activity.

A log plot of the percent remaining activity versus time for the non-aquated gold complex is presented in Figure 1, and first order kinetics are observed at all gold complex concentrations. Similar results were obtained for aquated gold complex samples (not shown). The reciprocals of the first order rate constants from these lines were plotted against the reciprocals of the gold halide concentrations, and the results are shown in Figure 2. The intercepts at the concentration axis ($1/K_I$) and at the rate constant axis ($1/k_p$) (see equation 2) were calculated, and the results are reported in

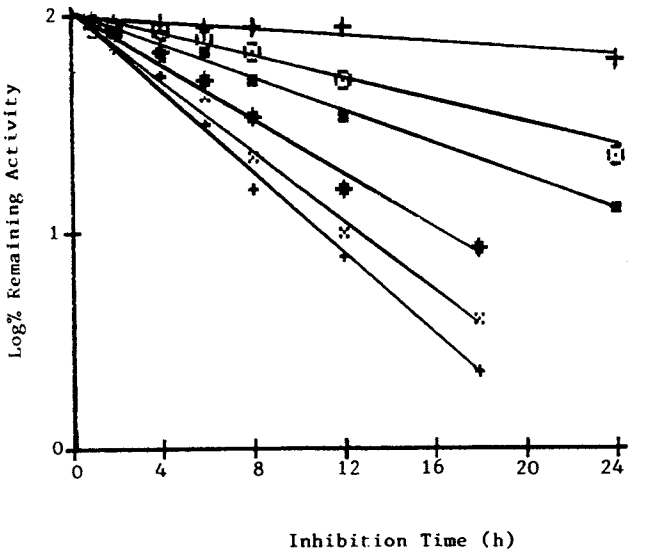

FIGURE 1 Inhibition of β-Glucuronidase by Non-Aquated $NaAuCl_4$. The log plot of the percent remaining activity obtained from the rate curves is measured against the inhibition time for several different gold concentrations. The concentration of the enzyme is 30 nM. All inhibition reactions take place in a 0.1 M phosphate buffer, pH 7.0, at 25°C. The enzyme activity was recorded at pH 9.5 at 37°C. The $NaAuCl_4$ concentrations are; -+- 0.5 μM, -□- 1.0 μM, -■- 1.5 μM, ♦ 2.0 μM, -×- 3.0 μM and -+- 4.0 μM. The control, not shown, exhibited no decrease in activity over the 24 h measuring period.

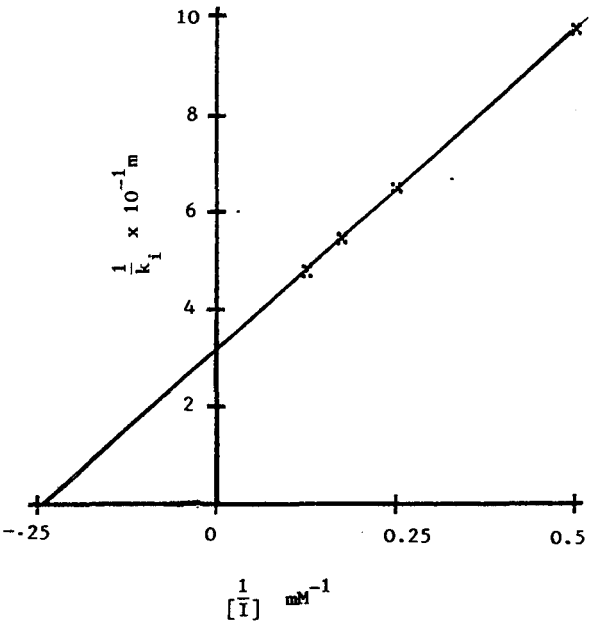

FIGURE 2 Plot of the Reciprocals of the Rate of Inactivation Verses the Reciprocal of the Non-Aquated $NaAuCl_4$ Inhibitor Concentration for β-Glucuronidase as described in equation 2. The rates were calculated from the slopes in Figure 1.

TABLE I
Compilation of Equilibrium and Rate Data Obtained from the Inhibition of β-Glucuronidase and Acid Phosphatase.

Gold Complex	Rate Experiments*		Equilibrium Experiments*
	$k_p 10^2 \text{min}^{-1}$	$K_I \mu M$	$K_I \mu M$ *(Inhibition Type)*
β-Glucuronidase			
Non-aq. $NaAuCl_4$	0.52	1.9	
Aq. $NaAuCl_4$	1.01	3.7	3.7 ± 0.1 (Mixed)
Non-aq. $KAuBr_4$	0.48	1.5	
Acid Phosphatase			
Non-aq. $NaAuCl_4$			
Fast reacting	50	8.5	
Slow reaction	1.0	5.0	
Aq. $NaAuCl_4$			
Fast reacting	46	12.5	
Slow reacting	2.1	9.8	1.1 ± 0.2 (Mixed)

* Calculation of activity measurement yields an average of ±5% error in the rate or equilibrium constants.

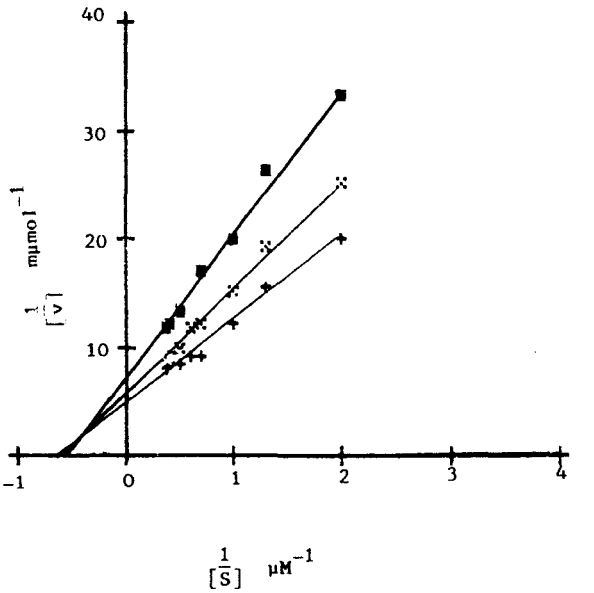

FIGURE 3 Lineweaver-Burk Double Reciprocal Plot of the Activity of the 24 h $NaAuCl_4$ Inhibited β-Glucuronidase Versus Substrate Concentration. The concentration of $NaAuCl_4$ inhibitor are; -■- 2.0 μM and - × - 1.0 μM. The uninhibited enzyme is represented by - + -.

Table I. A similar study was carried out (not shown) for the non-aquated $KAuBr_4$, and the results for K_I and k_p are also reported in Table I.

Lineweaver-Burk double reciprocal plots were made for the equlibrium inhibition by $NaAuCl_4$, and these results are shown in Figure 3. The K_I values for both the aquated and non-aquated samples are reported in Table I. The gold compound was observed to be acting as a mixed-type inhibitor.

Inhibition of Acid Phosphatase

Acid phosphatase was also inhibited by non-aquated and 24 h aquated $NaAuCl_4$ compounds. The gold concentrations ranged from 2.5 to 10.0 μM. The rate of inhibition was fairly rapid during a period of 5 to 10 min, and then it slowed considerably over the next hour. Further time-dependent inhibition (not shown) decreased only a negligable amount. The log plot for the non-aquated reaction is presented in Figure 4. There appears to be more than one inhibiting phosphatase species in contrast to the single species found in β-glucuronidase experiments. An analysis similar to that of Main[48] was carried out in order to identify the number of species in solution. The slower reacting one was found to yield a linear reaction. The rate was substracted from the rest of the curve, and the rates were replotted. The log replots of the corrected rates, illustrated in the inset of Figure 4, yielded first order reactions for the fast reacting enzyme. The reciprocals of the slope and inhibitor concentration were plotted for both acid phosphatase species (Figure 5 and 6), and

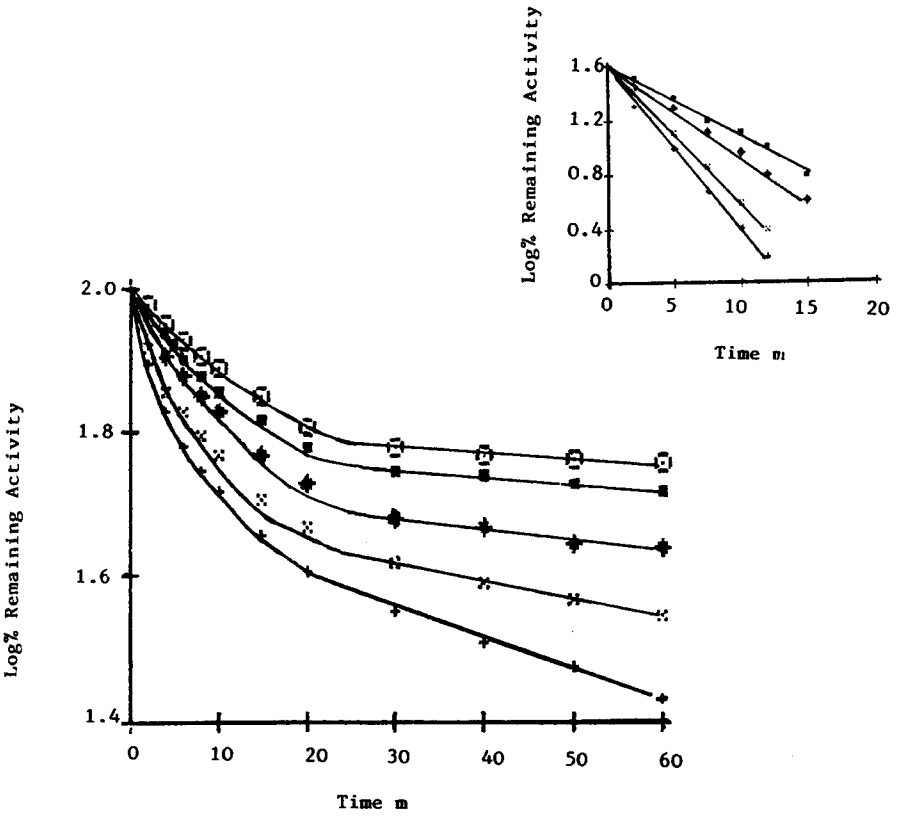

FIGURE 4 Inhibition of Acid Phosphatase by Non-Aquated $NaAuCl_4$. The semi-log plot of the percent of the remaining activity is plotted against the rate of inhibition for several concentrations of the gold compound. The curves indicate more than one inhibiting enzyme species. The slower reacting species which displayed first order kinetics was substracted from the more rapidly reacting form, and by use of linear regression analysis a single fast reacting species was then found to display first order kinetic (see insert). The inhibition experiments were carried out at pH 7.0 at 25°C while the activity measurements were run at pH 5.0 at 25°C. The enzyme concentration was 25 nM. The concentrations of $NaAuCl_4$. -⊡- 2.0 μM, -■- 2.0 μM, ✦ 3.5 μM, - × - 5.0 μM and - + - 10 μM. The control reaction in which the inhibitor was omitted did not yield any loss of activity over the time indicated (not shown).

the K_I and k_p values reported in Table I are for a fast and slow reacting species. A similar analysis was carried out for the aquated gold compound, and comparable curves were obtained (not shown). The K_p and k_p results are also reported in Table I.

A Lineweaver-Burk double reciprocal plot was prepared on two lower $NaAuCl_4$ inhibitor concentrations, and the results are shown in Figure 7. The inhibition reached equilibrium within 24 h. The K_I values are reported in Table I, and the curves reflect a mixed-type inhibition.

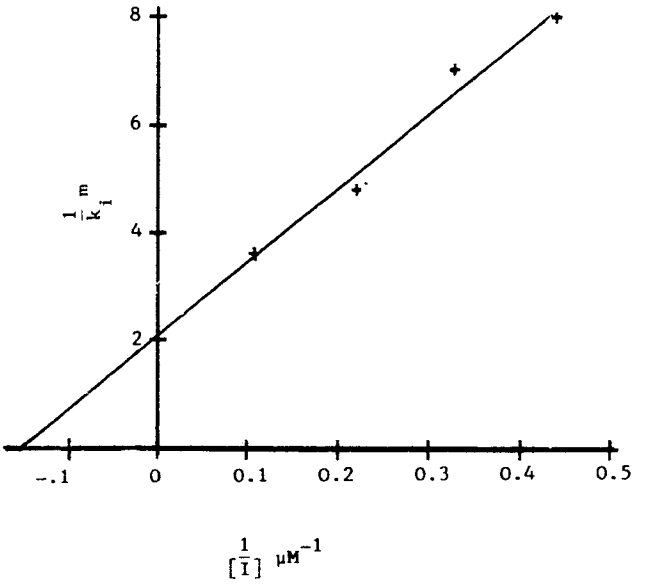

FIGURE 5 Plot of the Reciprocal of the Rate of Inhibition of the Fast Reacting Acid Phosphatase Species versus the Reciprocal of the Non-Aquated $NaAuCl_4$ Inhibitor Concentrations as described in equation 2. The rates were calculated from the slopes in Figure 4.

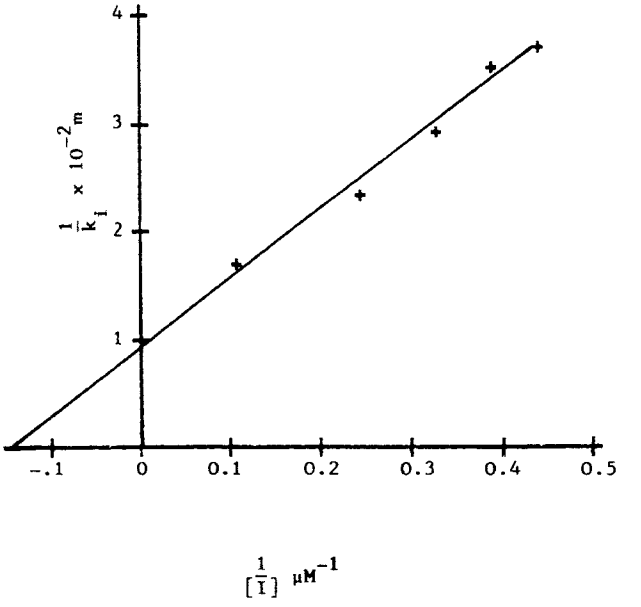

FIGURE 6 A similar plot to Figure 5 but for the Slow Reacting Acid Phosphatase species.

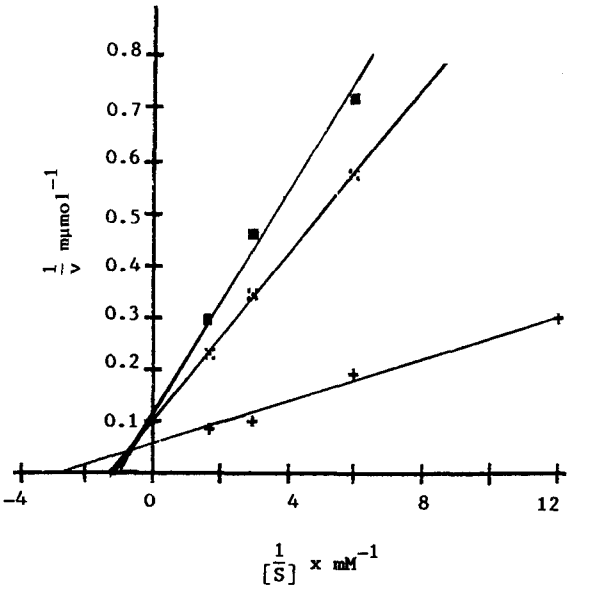

FIGURE 7 Lineweaver-Burk Double Reciprocal Plot of the Activity of the 24 h $NaAuCl_4$ Inhibited Acid Phosphatase Species Versus Substrate Concentration. Concentrations of $NaAuCl_4$ inhibitor were, −■− 1.32 μM and − × − 1.15 μM. The uninhibited enzyme is represented by − + −.

DISCUSSION

The choices of bovine liver β-glucuronidase and wheat germ acid phosphatase as models for the measurement of inhibition by gold compounds of enzymes appears to be acceptable since these enzymes are similar in nature to the synovial type and are easy to obtain and purify. The gold compound, although not used as an anti-inflammatory drug, shows a marked degree of inhibitory behavior and allows us to use it as a barometer against the gold drug complexes.

The purification of the enzymes yield results similar to previous workers. The use of phenolphthaleinglucuronate to locate the β-glucuronidase on the gel proved to be advantageous, but the red phenolphthalein band tended to spread rapidly and thus color development at high pH had to be performed rapidly after the enzyme reaction was stopped. Both enzymes were stable for several weeks at 4°C.

Gold halides are reactive towards proteins in aqueous solution, however, one or more of the halo ligands are slowly displaced when dissolved in an aqueous medium. At pH 7.0 the predominantly stable species are $AuCl_3OH^-$ and $AuCl_2(OH)_2^-$. In the present study the aquated K_I values for both enzymes, determined from the rate studies, proved to be larger than those in the non-aquated studies. This may be reflected either in the greater ease of removal of the halo ligand than the hydroxy one by a neutrophilic group on the enzyme, or due to the greater degree of halogenicity by the tetrahalo compound, providing more sites of attack by the enzyme.

The small values of k_p in the β-glucuronidase experiments suggest that the initial inhibition is due primarly to the equilibrium association between the enzyme and the

gold compound. When the experiments were carried out at lower gold concentrations the equilibrium constant obtained by a double reciprocal plot was comparable to those from the rate experiments. This supports the contention that the covalent bond formation is very slow and does not significantly effect the more rapid equlibrium which also leads to the inhibition of the enzyme.

Comparison between $AuCl_4^-$ and $AuBr_4^-$ in rate experiments indicate that there is little if any difference in the equilibrium or rate of covalent bond formation upon substitution of the halide atom. Thus, inhibition is not sensitive to small size or lability differences of the ligands.

The acid phosphatase inhibition experiments indicate that two enzyme species are present. A number of investigators have reported[49-51] that the wheat germ source contained several isoenzymes; as many as four have been suggested. Resolution of our data yielded two reacting species. The K_I values from the rate experiments for both enzymes produced comparable values with a slightly more stable one for the slower reacting enzyme, and as has been observed in the β-glucuronidase experiments, the aquated form is slightly less stable. The main difference between the two forms appears to be the one hundred fold increase in the rate constant k_p for the faster reacting form. This value is also significantly greater those found in the β-glucuronidase studies. It is quite possible that a highly reactive nucleophilic group such as a sulfhydryl is the main locus for attachment to the gold atom. It has been previously reported[52] that some acid phosphatases are inhibited by sulfhydryl reagents, and that at least one isoenzyme from wheat germ acid phosphatase has a cysteinyl side chain near the active site histidine residue.[51] The ten fold increase for the K_I in the rate experiments is probably due to the high degree of stability bestowed by the more rapidly reacting covalent bond formation leading to a non-equilibrium state which comprises a mixture of the two enzyme forms. A longer time study would have yielded continued decreasing K_I values, however, instability of the enzyme under experimental conditions precluded further investigation along these lines.

In conclusion, it appears that the gold (III) halo compounds bind tightly to the enzymes and that it is most likely this characteristic that precludes them from being effective drugs. These compounds appear to be able to discriminate reactive enzyme forms depending upon the particular side chains available for bonding. It is not likely that any significant oxidation is occurring since the large excess of gold compound would most likely produce, more rapid and complete inactivation. Previous oxidation studies on simple compounds such as NADH, or proteins such as RNAase and lysozyme, have yielded precipitates. Yet, this mode of action, at least in part, cannot be completely ruled out.

The study in the following paper is directed towards those gold compounds which are presently being used as anti-inflammatory drugs in order to determine whether the above binding properties will be useful in distinguishing their pharmacological effects.

References

1. Persellin, R.H. (1968) *Med. Clin, N. Am.* **52,** 635.
2. Chayen J. and Bitensky, L. (1971) *Ann. Rheum. Dis.* **30,** 522.
3. Levy, G.A. and Conchie, J. (1966) In *Glucuronic Acids*, Button, G.J. ed., pp. 301. New York Acad. Press.
4. Jacox R.F. and Feldman, A. (1955) *J. Clin. Invest.*, **34,** 263.
5. Caygill J.C. and Pitkeathy, D.A. (1966) *Ann Rheum Dis.* **25,** 137.
6. Forestier, J. (1929) *Bull. Mem. Soc. Med. Hop. Paris,* **53,** 323.

7. Ragan, C.(1951) *Bull. N.Y. Acad. Med.*, **27,** 63.
8. Short, C.L. Beckman W.W. and Baner, W.(1964) *New Eng. J. Med.*, **235,** 362.
9. Freyberg, R.H. (1960) *in Arthritis,* Hollander, J.L. ed., Ch. 19. Philadelphia, Lea and Febiger.
10. Ellman, P., Lawrence, J.S. and Thorold, G.P.(1940) *Brit. Med. J.*, **2,** 314.
11. Fraser, T.N. (1945) *Ann. Rheum. Dis.*, **4,** 71.
12. Waine, H., Baker, F. and Mettier, S.R. (1947) *Calif. Med.*, **66,** 295.
13. Sadler, P.J.(1976) *Struct. Bonding (Berlin)*, **29,** 171.
14. Shaw, C.F. (1979) *Inorg. Persp. Biol.*, **2,** 287.
15. Brown D.H. and Smith, W.E. (1980) *Chem. Soc. Rev.*, **9,** 217.
16. Stone, K.H., Mather, S.J. and Gibson, P.P. (1975) *Prostaglandins,* **10,** 241.
17. Newcomb, D.S. and Ishikawa, Y. (1976) *Prostaglandins,* **12,** 849.
18. Pennys, N.S., Ziboh, V., Gottlieb, N.L. and Katz, J. (1974) *J. Invest. Dermatol,* **63,** 356.
19. Adam, M., Deyl, Z., and Rosmus, J. (1966). *J. Med. Pharmacol. Exp.* **14,** 129.
20. Adam, M. and Kuhn, K. (1968) *Eur. J. Biochem.*, **3,** 407.
21. Deyl, Z., Rosmus, J. and Adam, M. (1970) *Eur. J. Biochem.*, **13,** 589.
22. Measel, J.W. (1975). *Infect. Immunol.*, **11,** 350.
23. Finkelstein, A.E., Rossman, R.R. and Walz, D.T. (1973) *Inflammation,* **2,** 143.
24. Persellin, R.H., Hess, E.V. and Ziff, M. (1967) *Arthritis Rheum.*, **10,** 99.
25. Ennis, R.S., Granada, J.L. and Posner, A.S. (1968) *Arthritis Rheum.*, **11,** 756.
26. Weissman, G., Zurier, R.B. and Spieler, P.Z. (1971) *J. Exp. Med.*, **134,** 521.
27. Dimartino M.J. and Walz, D.T. (1972) *Inflammation,* **2,** 131.
28. Janoff, A. (1968) *Biochem. Pharmacol.*, **19,** 626.
29. Ghadially F.N. and Lalonde, J.M.A. (1978) *Virchows Arch. B.*, **28,** 31.
30. Persellin, R.H. and Zeff, M. (1966) *Arthritis Rheum.*, **9,** 57.
31. Lorber, A., Bovy, R.A. and Chang, C.C. (1972) *Nature (Lond.)* **235,** 250.
32. Lorber, A., Atkins, C., Chang, C.C. and Starrs, J. (1970) *Arthritis Rheum.*, **13,** 333.
33. Thompson, R.H.S. and Whittakew, V.P. (1941) *Biochem. J.*, **41,** 342.
34. Danpure, C.J. (1976) *Biochem. Trans.*, **4,** 161.
35. Friedman, M.E., Chasteen, L., Shaw, R., Allen, S. and McAuliffe, C.A. (1982) *Biol. Inter.*, **42,** 321.
36. Plapp, B.V. and Cole, R.D. (1966) *Archs. Biochem. Biophys.*, **116,** 193.
37. Stahl, P.D. and Touster, O. (1971) *J. Biol. Chem.*, **246,** 5398.
38. Plapp, B.V. and Cole, R.D. (1967) *Biochemistry,* **6,** 3676.
39. Kitz, R. and Wilson, I.B. (1962). *J. Biol. Chem.*, **237,** 3245.
40. Main, A.R. (1964) *Science,* **144,** 992.
41. Bernfield, P. Nisselbaum, J.S. and Fishman, W.H. (1953) *J. Biol. Chem.*, **202,** 763.
42. Talalay, P., Fishman, W.H. and Huggins, C. (1946) *J. Biol. Chem.*, **166,** 757.
43. Fishman, W.H., Springer, B. and Brunette, R. (1948) *J. Biol. Chem.*, **173,** 449.
44. Brindenberger, H., and Hanson, R. (1953) *Helv. Chim. Acta,* **36,** 900.
45. Hofstee, B.H.J. (1954) *Archs. Biochem. Biophys.*, **51,** 139.
46. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.*, **193,** 265.
47. Laemmli, U.K. and Maizel, R. (1970) *Nature (Lond.)* **227,** 680.
48. Main, A.R. (1969) *J. Biol. Chem.*, **244,** 829.
49. Joyce, B.K. and Grisolia, S.J. (1960) *J. Biol. Chem.*, **235,** 2278.
50. Verjee, Z.H.M. (1969) *Eur. J. Biochem.* **9,** 459.
51. Hickey, M.E. (1976) Thesis, Purdue Univ., *Univ. Microfilms Internat.*, Ann Arbor, MI.
52. Puddenphatt, R.J. (1978) in *The Chemistry of Gold,* Monograph 16, pp. 182–183. Elsevier.