Biochemical Pharmacology, Vol. 23, pp. 153–162. Pergamon Press, 1974. Printed in Great Britain.

# THE EFFECT OF GOLD SALTS ON THE BIOSYNTHESIS OF URIDINE NUCLEOTIDES IN HUMAN GRANULOCYTES

WENDY J. WESTWICK, JENNIFER ALLSOP and RICHARD W. E. WATTS

Division of Inherited Metabolic Diseases, Medical Research Council Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, England

(*Received* 6 *March* 1973; *accepted* 25 *June* 1973)

**Abstract**—The action of sodium aurothiomalate on the activity of the enzymes which catalyse the synthesis of the uridine 5′-phosphates has been studied *in vitro* using normal human granulocytes. The following enzymes were markedly inhibited in disrupted cell preparations: carbamoyl phosphate: L-aspartate transferase (EC 2.1.3.2), orotidine-5′-phosphate-pyrophosphate phosphoribosyltransferase (EC 2.4.2.10) and uridine mono- and diphosphokinases [ATP: nucleosidemonophosphate phosphotransferase (EC 2.7.4.4) and ATP:nucleosidediphosphate phosphotransferase (EC 2.7.4.6)]. ATP: uridine 5′-phosphotransferase (EC 2.7.1 f) was also inhibited although to a lesser extent than the other enzymes. The activities of L-4,5-dihydroorotate aminohydrolase (EC 3.5.2.3), L-4,5-dihydroorotate: $O_2$ oxidoreductase (EC 1.3.3.1) and orotidine-5′-phosphate carboxylase (EC 4.1.1.23) were not affected under the conditions used. The inhibitory effects could be prevented by adding sulphydryl group-protecting reagents (2-mercaptoethanol, L-cysteine, dithiothreitol and reduced glutathione), the inhibition was reduced slightly by adding serum to the system. There was no inhibition when intact cells were used. Some preliminary observations on the possible relationship of the gold-sensitive sulphydryl groups to the active sites of enzymes have also been made. The possible implications of these findings in connection with gold induced neutropoenia are discussed.

GOLD salts such as sodium aurothiomalate (Myocrisin) are beneficial in the treatment of rheumatoid arthritis.[1] However, one of the hazards of therapy is the unpredictable occurrence of neutropenia in some patients despite regular haematological surveillance. The work of Denman and Denman[2] using the lymphocyte transformation test has shown that an immune mechanism may be responsible in some cases. But in other patients a biochemical abnormality could be the cause of the neutropenia. For instance, any interference either direct or indirect with nucleic acid synthesis in the bone marrow of a patient abnormally sensitive to gold would impair cell production and could lead to agranulocytosis.

The present studies were made using peripheral blood granulocytes which are the mature end cells of the tissue system upon which the drug exerts its toxic action *in vivo*. Peripheral granulocytes are more conveniently obtained from normal healthy volunteers than bone marrow samples and have the advantage that a pure cell population is easily prepared from venous blood. All of the enzymes of the *de novo* synthesis,[3] salvage and interconversion pathways of uridine nucleotide production are present in mature circulating granulocytes although their activity is less than in bone marrow cells.*

* J. Allsop and W. J. Westwick, unpublished data.

Gold is a heavy metal and would be expected to inhibit enzymes whose activity depends on the presence of sulphydryl groups. This paper reports the results of an investigation of the effect of sodium aurothiomalate on the following eight enzymes in disrupted and intact granulocyte preparations:

(1) Aspartate transcarbamylase (carbamoylphosphate: L-aspartate transferase, EC 2.1.3.2).
(2) Dihydroorotase (L-4,5-dihydroorotate aminohydrolase, EC 3.5.2.3).
(3) Dihydroorotate dehydrogenase (L-4,5-dihydroorotate: $O_2$ oxidoreductase, EC 1.3.3.1).
(4) Orotate phosphoribosyltransferase (orotidine-5′-phosphate: pyrophosphate phosphoribosyltransferase, EC 2.4.2.10).
(5) OMP decarboxylase (orotidine-5′-phosphate carboxylase, EC 4.1.1.23).
(6) Nucleoside monophosphate kinase (ATP: nucleosidemonophosphate phosphotransferase, EC 2. 7.4.4).
(7) Nucleoside diphosphate kinase (ATP: nucleosidediphosphate phosphotransferase, EC 2.7.4.6).
(8) Uridine kinase (ATP: uridine 5′-phosphotransferase, EC 2.7.1.f).

The relevant metabolic pathway is shown in Fig. 1.* The modifying effect of serum proteins on the action of sodium aurothiomalate has also been studied.

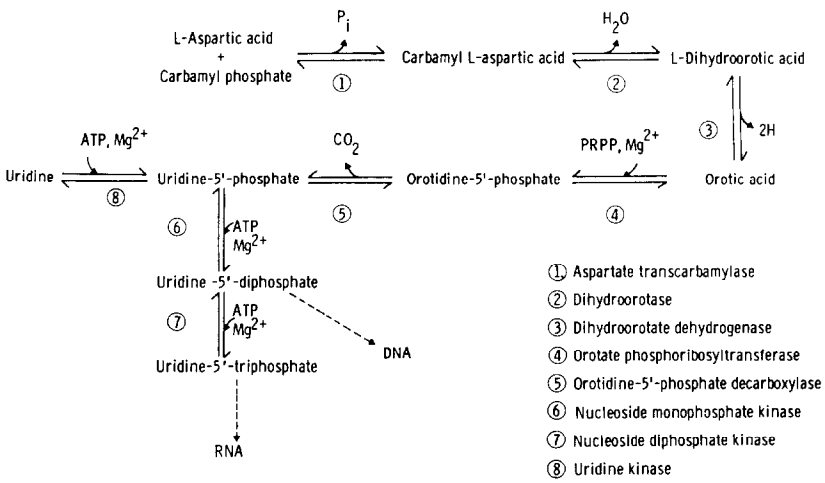


FIG. 1. Enzymes involved in the *de novo*, "salvage" and interconversion pathways of uridine nucleotide synthesis.

## METHODS AND MATERIALS

All radioactive substrates, except [6-$^{14}$C]DL-dihydroorotic acid and [6-$^{14}$C]OMP, were obtained from the Radiochemical Centre, Amersham. [6-$^{14}$C]DL-dihydroorotic acid was synthesized by the hydrogenation of [6-$^{14}$C]orotic acid in the presence of a rhodium catalyst.[4] [6-$^{14}$C]OMP was prepared using the method of Lieberman *et al.*[5]

* The following abbreviations have been used: ATP, adenosine 5′-triphosphate; OMP, orotidine 5′-monophosphate; UMP, uridine 5′-monophosphate; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate; PRPP, α-D-ribofuranose-1-pyrophosphate-5-phosphate ("phosphoribosylpyrophosphate").

The di-magnesium salt of PRPP was purchased from P-L Ltd. and the other biochemicals from Sigma (London) Chemical Co. Ltd. Pure crystalline sodium aurothiomalate (CH(SAu)COONa $CH_2COONa$) was a gift from May & Baker, Ltd. The 30% bovine albumin solution used in the separation of granulocytes was obtained from Stayne Laboratories Ltd. All solutions were made up in deionized water.

*Separation of granulocytes from blood.* Blood samples were taken from normal healthy volunteers and the granulocytes were isolated by a method employing dextran sedimentation followed by differential centrifugation through a solution of bovine albumin.[3] The washed granulocytes were finally resuspended in isotonic saline (usually 0·8 ml containing 5–10 $\times$ $10^7$ cells from 40 ml blood). In the studies on the effect of sodium aurothiomalate on whole cells, the cells were suspended in autologous serum/Seligman's[6] balanced salt solution [1:1 (v/v)]. This medium caused less clumping of cells than serum/Hanks's solution.

*Disruption of the cells.* The granulocytes were disrupted by ultrasonic vibrations prior to assay as described previously.[3]

*Protein determinations.* The protein content of the sonicated cell preparations was measured colorimetrically.[7]

*Enzyme assays.* The micro-radiochemical methods used to assay aspartate transcarbamylase, dihydroorotase, dihydroorotic dehydrogenase, orotate phosphoribosyltransferase and OMP decarboxylase were described previously.[3]

*Uridine kinase.* This enzyme was measured using a modification of the method of Furlong.[8] The assay mixture, total volume 100 $\mu$l, contained 70 $\mu$M [2-$^{14}$C]uridine (0·4 $\mu$Ci), 4 mM ATP, 5 mM $MgCl_2$, 0·1 M tris-HCl (pH 8·0) and granulocytes (0·1–0·3 mg protein) which had been sonicated for 10 sec. The system was incubated for 30 min at 37° before stopping the reaction with $HClO_4$ (25 $\mu$l, 15% w/v solution).

The samples were cleared by centrifugation before chromatographing on Whatman DE81 ion-exchange paper. Portions of the samples (25 $\mu$l) were co-chromatographed with non-radioactive UMP, UDP and UTP (0·3 $\mu$mole of each) since the initial product, [$^{14}$C]UMP, is further phosphorylated to the di- and tri-phosphate nucleotides by the uridine nucleotide kinases also present. Descending chromatography with ammonium formate solution (0·025 M) for 4 hr separated uridine from the nucleotides. The amount of $HClO_4$ present caused only a very slight degree of streaking in this chromatographic system. The nucleotides were located by their quenching of u.v. light (254 nm), the relevant areas of paper cut out and their radioactivity determined by liquid scintillation counting.[3]

*Uridine mono-and-diphosphokinases.* These two enzymes were assayed as a single step using a method based on that of Lieberman *et al.*[9] The total volume of the assay system was 100 $\mu$l and it contained 40 $\mu$M [4-$^{14}$C]UMP (0·2 $\mu$Ci), 4 mM ATP, 5 mM $MgCl_2$ 0·02 M phosphate buffer (pH 7·5) and granulocytes (0·10–0·25 mg protein) sonicated for 8 sec. The tubes were incubated for 2 min at 37° and then $HClO_4$ (50 $\mu$l, 15% w/v) solution was added to stop the reaction.

After removing the precipitated protein by centrifugation, [$^{14}$C]UDP and [$^{14}$C]UTP were separated from [$^{14}$C]UMP using high voltage electrophoresis on 3 MM paper. Twenty-five $\mu$l of sample were co-electrophoresed with 0·3 $\mu$mole of non-radioactive UDP and UTP. The system used was 0·015 M citrate at pH 5·05 and electrophoresis was continued for 25 min at 130 V/cm. The $^{14}$C-nucleotides were located and counted as described previously.

*The effect of sodium aurothiomalate on disrupted granulocyte preparations.* The effect of a range of concentrations of sodium aurothiomalate (final concentrations 0·001–1·0 mM) on the assay of these pyrimidine synthesizing enzymes present in sonicated cell preparations was tried. The drug was dissolved in either (a) water or (b) autologous serum [75% (v/v)] and added directly to the assay medium. The cell preparations were not incubated with the drug prior to assay since early experiments had shown this was not necessary.

The sulphydryl group protecting agents 2-mercaptoethanol, dithiothreitol, L-cysteine and reduced glutathione were used at a final concentration of 5 mM in the incubation medium. They were dissolved in water and the pH of the solution adjusted to that of the assay system. This solution was added to the disrupted granulocytes about 10 min before the assay, the concentration at this stage being 9–25 mM.

Experiments were also carried out using another gold salt, sodium chloroaurate ($NaAuCl_4$) and a known sulphydryl group inhibitor, *p*-hydroxymercuribenzoate. These two compounds were each used as an aqueous solution, the pH of which was adjusted to that of the assay system. Their concentrations were similar to those of sodium aurothiomalate.

*The effect of pre-incubation of enzyme with a substrate.* In other experiments, the buffered disrupted granulocytes were pre-incubated for 10 or 20 min with each substrate before adding the sodium aurothiomalate (final concentration 0·1 mM). The enzyme reaction was then started by addition of the other substrate. It was necessary to do control assays in a similar manner with water added instead of gold salt solution.

*The incubation of intact granulocytes with sodium aurothiomalate.* The cells were suspended in 50% (v/v) autologous serum in Seligman's balanced salt solution (approx. 2–3 $\times$ $10^7$ cells/ml) containing 0, 0·1 or 1·0 mM sodium aurothiomalate. One ml aliquots of these suspensions were incubated at 37° for 2 hr. At the end of this period the cells were separated by centrifugation (100 *g*, 4 min, 4°) and quickly washed twice with 5 ml of ice-cold, aqueous sodium chloride [0·85% (w/v)] solution. The washed cell pellet was resuspended in 0·35 ml of aqueous sodium chloride [0·85% (w/v)] solution and the viability checked by the trypan blue method.[10] The cells were then sonicated and the enzymes assayed. Protein analyses were done to correct for any differences in cell concentration in the three cell suspensions.

## RESULTS

Sodium aurothiomalate inhibited aspartate transcarbamylase, orotate phosphoribosyltransferase and the uridine mono- and diphosphate kinases. The dose–response curves (Figs. 2–4) varied only slightly between different individuals. However, with each of these three enzymes the dose–response curves in the presence of serum showed less inhibition than when purely aqueous media were used. Serum alone depressed the activity of orotate phosphoribosyltransferase by approximately 50%, but appeared to have no effect on the activity of any of the other enzymes. The gold salt dose–response curves in serum relate to the non-drug control sample which contained serum.

Sodium chloroaurate and *p*-hydroxymercuribenzoate inhibited the same enzymes in the same range of concentrations as sodium aurothiomalate

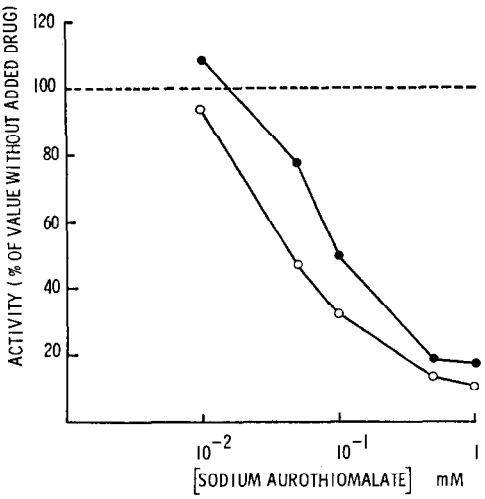


FIG. 2. The *in vitro* inhibition of aspartate transcarbamylase by aqueous sodium aurothiomalate (○) and the modifying effect of the presence of autologous serum (●). The substrate concentrations were: 15 mM L-aspartate and 10 mM carbamylphosphate.

Addition of 5 mM concentrations of 2-mercaptoethanol, dithiothreitol, L-cysteine and reduced glutathione protected against inhibition equally effectively in all cases. These thiol group protecting agents had a slightly stimulatory effect on the enzyme activities when added to the assay system in the absence of gold salt.

Uridine kinase was also inhibited by sodium aurothiomalate, although less effectively than the previously mentioned enzymes (Fig. 5). This inhibition was little affected by the presence of serum and could be partly overcome by 2-mercaptoethanol.

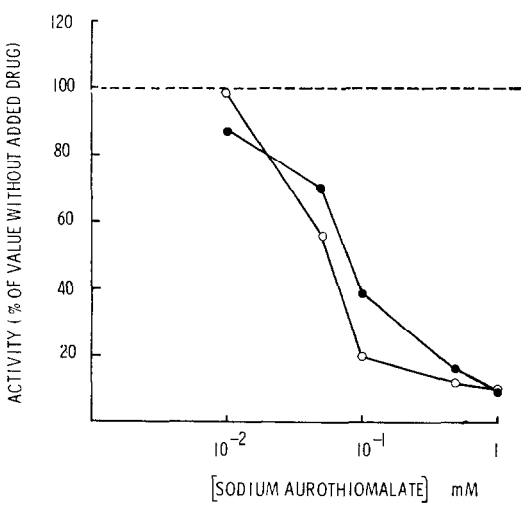


FIG. 3. The *in vitro* inhibition of orotate phosphoribosyltransferase by aqueous sodium aurothiomalate (○) and the modifying effect of the presence of autologous serum (●). The substrate concentrations were: 60 $\mu$M orotic acid, 1·0 mM PRPP and 2·5 mM $Mg^{2+}$.

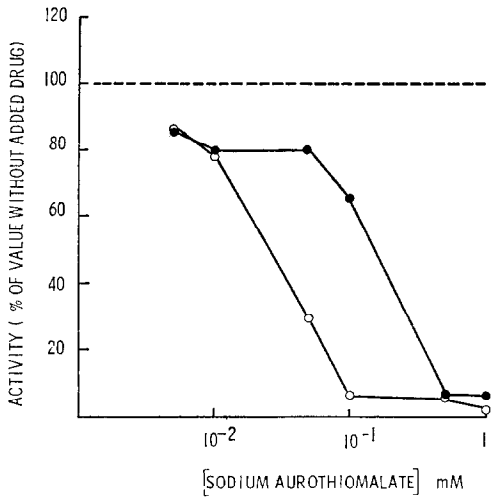


FIG. 4. The *in vitro* inhibition of nucleoside mono and diphospho kinases by aqueous sodium urothiomalate (○) and the modifying effect of the presence of autologous serum (●). The substrate concentrations were: 40 $\mu$M UMP, 4·0 mM ATP and 5·0 mM $Mg^{2+}$.

There was little effect of sodium aurothiomalate on either dihydroorotase or OMP decarboxylase, 1·0 mM sodium aurothiomalate inhibiting dihydroorotase by only 20%.

Sodium aurothiomalate (0·05–0·1 mM) slightly stimulated dihydroorotic dehydrogenase. This effect though variable in extent was found repeatedly with the granulocytes from different normal subjects (Fig. 6). Sodium chloroaurate had a similar effect.

Preincubating the disrupted granulocyte preparations with PRPP–$Mg_2$, but not with orotic acid, reduced the susceptibility of the orotate phosphoribosyltransferase

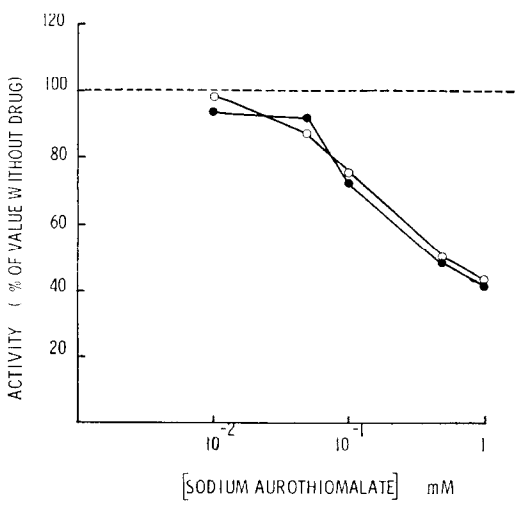


FIG. 5. The *in vitro* inhibition of uridine kinase by aqueous soidum aurothiomalate (○) and the modifying effect of the presence of autologous serum (●). The substrate concentrations were: 70 $\mu$M uridine, 4·0 mM ATP and 5·0 mM $mMg^{2+}$.

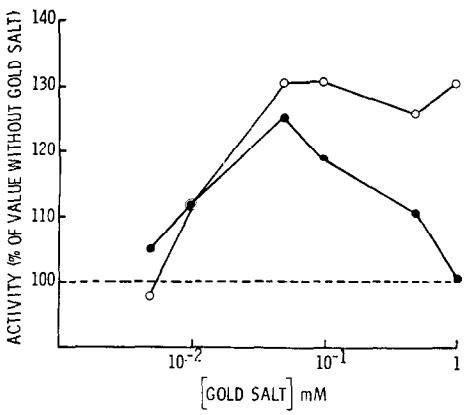


FIG. 6. The effect of aqueous sodium aurothiomalate (●) and sodium chloroaurate (○) on the activity of dihydroorotic dehydrogenase. The concentration of L-dihydroorotic acid was 0·5 mM.

activity to gold inhibition by about one half. The nucleoside mono- and diphosphokinases were also partially protected by preincubation with UMP, but this effect was not shared by ATP (Table 1). The inhibitory effect of gold salts on the aspartate transcarbamylase activity of disrupted granulocytes was unaffected by preincubation with either L-aspartate or carbamylphosphate.

TABLE 1. THE EFFECT OF PRE-INCUBATING DISRUPTED GRANULOCYTES WITH A SUBSTRATE ON THE ENZYME INHIBITION BY SODIUM AUROTHIOMALATE (0·1 mM)

| Enzyme | Pre-incubation | | Activity (% of value without drug) | | |
|---|---|---|---|---|---|
| | Substrate | Time (min) | Experiment No. (1) | (2) | (3) |
| Orotate phosphoribosyl transferase | None | 10 | — | 24 | 11 |
| | Orotic acid | 10 | 34 | 23 | 12 |
| | PRPP.$Mg_2$ | 10 | 79 | 51 | 21 |
| Nucleoside mono and diphosphokinases | None | 10 | <1 | <1* | <1* |
| | UMP | 10 | 8 | 7* | 7* |
| | ATP | 10 | <1 | <1* | — |

* 0·05 mM sodium aurothiomalate used.

TABLE 2. THE EFFECT OF INCUBATING INTACT GRANULOCYTES WITH SODIUM AUROTHIOMALATE ON THE SUBSEQUENT ASSAY OF PYRIMIDINE SYNTHESIZING ENZYMES

| Enzyme | % Activity* Concn. of gold in medium (mM) 0 | 0·1 | 1·0 |
|---|---|---|---|
| Aspartate transcarbamylase | 100 | 93 | 93 |
| Orotate phosphoribosyltransferase | 100 | 104 | 104 |
| Uridine kinase | 100 | 98 | 94 |
| UMP/UDP kinase | 100 | 125 | 97 |

* Corrected for any differences in protein content in the assays.

The enzymes were not inhibited by incubating the intact cells with sodium aurothiomalate (0·1 and 1·0 mM) for 2 hr before they were disrupted and the enzymes assayed (Table 2). This procedure caused only slight clumping of the cells and >90% of the cells were viable as judged by the trypan blue exclusion test.

## DISCUSSION

Heavy metals inhibit the activity of sulphydryl group containing enzymes. The present study has shown that human granulocyte aspartate transcarbamylase, orotate phosphoribosyltransferase and uridine mono- and diphosphokinases were markedly inhibited by this drug *in vitro*. The results of the experiments with 2-mercaptoethanol, dithiothreitol, L-cysteine and reduced glutathione indicate that the inhibition is due to the binding of gold to the enzyme sulphydryl groups. Additional evidence is given by the findings that sodium chloroaurate and *p*-hydroxymercuribenzoate are also inhibitory. It is noteworthy that a relationship between the level of reduced glutathione which is a naturally occurring sulphydryl group protecting agent and the occurrence of granulocytopoenia was suggested as early as 1936 by Parker and Kracke.[11]

The occurrence of enzyme inhibition *in vivo*, will depend on physiological factors which influence the local concentration of the metal. Recent work[12,13] has shown that most of the circulating gold is bound to plasma albumin. Our observation that sodium aurothiomalate is less toxic *in vitro* in the presence of serum suggests that gold–protein binding may play a role in the prevention of toxic effects *in vivo*.

It would be of interest to know if the thiol groups of the enzymes which are susceptible to inhibition by gold salts are situated at catalytic sites on the enzyme molecule. The protection against gold-inhibition which preincubation with PRPP–$Mg_2$ affords to orotate phosphoribosyltransferase suggests that the thiol groups are at or near the PRPP binding site in this. Conversely, the results of preincubation with orotic acid suggests that they are not related to the pyrimidine binding site. Similarly, the present results suggest that the thiol groups may be related to the UMP binding sites in the UMP/UDP kinases, although it was not possible with the present techniques to show whether one or both of the kinases were involved. The aspartate transcarbamylase in microorganisms is known to be a thiol group containing enzyme, and most of these groups are associated with the regulatory rather than the catalytic subunit.[14] If the human enzyme is similar, this location of thiol groups may explain why pre-incubating the enzyme with substrate did not protect against inhibition by gold in our experiments. It should be emphasized that studies of this type with unpurified enzymes must be interpreted with caution, therefore the present conclusions concerning the location of the thiol groups to which the gold binds are only tentative.

It seems likely that the therapeutic action of gold salts is related to combination of the metal with enzyme sulphydryl groups. Thus, Persellin and Ziff[15] have suggested that the beneficial effect of gold therapy may be due to the inhibition of lysosomal enzymes, such as acid phosphatase (EC 3.1.3.2) and β-glucuronidase (EC 3.2.1.31), which have a role in the propagation of inflammation. Gold (0·5–5·0 mM) has also been reported to inactivate granulocyte elastase (EC 3.4.4.7).[16] However, these effects only occurred at gold concentrations which were very much higher than the plasma gold levels observed during therapy, so that concentration of gold by phagocytic

cells has to be postulated. It should be emphasized that some of the enzymes involved in uridine nucleotide synthesis can be significantly inhibited by much lower gold concentrations (0·05–0·1 mM) (Figs. 2–4) which are similar to the total concentration of plasma gold found during therapy (approximately 0·015–0·09 mM depending on time after injection). Attempts to correlate plasma total gold levels with therapeutic benefit and the occurrence of side effects have given contradictory results.[17,18] Further work is needed to relate the ratio of protein bound and non protein bound gold to the outcome of treatment, and to clinically important gold toxicity.

Another factor influencing the *in vitro* effects of gold salts may be the concentration of gold by various tissues, and within different subcellular organelles. Our work on intact granulocytes suggests that these cells do not take up sodium aurothiomalate at all readily *in vitro* possibly due to interference with membrane sulphydryl groups, but bone marrow cells may behave differently. Jessop *et al.*[19] have found gold in the polymorphs from experimentally produced sterile inflammatory exudates of patients during gold therapy. Gottlieb and his colleagues[20] measured the gold content of post-mortem tissues from a patient who had been on aurothioglucose therapy for several years (total gold administered was 2·53 g) and they present some evidence for the accumulation of gold in the bone marrow. The studies of Persellin and Ziff[15] showed that guinea pig macrophages which are very actively phagocytic and pinocytic readily took up sodium aurothiomalate from the incubating medium *in vitro*.

The present work has identified the points on the uridine nucleotide biosynthetic pathways which are sensitive to inhibition by gold salts. It seems likely that the clinical significance of this susceptibility will be influenced by factors which affect the intracellular and extracellular availability, concentration and distribution of gold *in vivo*. The binding of gold to plasma proteins is one such factor which merits further study.

The supply of uridine nucleotides is only one determinant of bone marrow cell replication and differentiation. Thus, there is now good evidence[21] that a humoral colony stimulating factor, which appears to be produced by macrophages[22,23] regulates the rate of leukopoeisis. The possibility that gold salts could act at this level of physiological organization rather than, or as well as, on biosynthetic mechanisms has to be considered.

*Acknowledgements*—The authors would like to thank David Fyfe and Robert Grimes (sandwich student) for their technical assistance.

## REFERENCES

1. Empire Rheumatism Council, Research Subcommittee, *Ann. rheum. Dis.* **19,** 95 (1960).
2. E. J. Denman and A. M. Denman, *Ann. rheum. Dis.* **27,** 582 (1968).
3. W. J. Westwick, J. Allsop and R. W. E. Watts, *Biochem. Pharmac.* **21,** 1955 (1972).
4. M. Green and S. S. Cohen, *J. biol. Chem.* **225,** 397 (1957).
5. J. Lieberman, A. Kornberg and E. S. Simms, *J. biol. Chem.* **215,** 403 (1955).
6. P. Grabar, N. M. Seligman and J. Bernard, *Ann. Inst. Pasteur.* **88,** 548 (1955).
7. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
8. N. B. Furlong, *Analyt. Biochem.* **5,** 515 (1963).
9. J. Lieberman, A. Kornberg and E. S. Simms, *J. biol. Chem.* **215,** 429 (1955).
10. J. H. Cutts, in *Cell Separation Methods in Haematology*, p. 182. Academic Press, New York (1970).
11. J. P. Parker and R. R. Kracke, *Am. J. clin. Path.* **6,** 41 (1936).
12. R. Eberl, in *Rheumatoid Arthritis* (Eds. W. Müller, H.-G. Harwerth and K. Fehr), p. 617. Academic Press, New York (1971).

13. B. R. MASCARENHAS, J. L. GRANADA and R. H. FREYBERG, *Arthr. Rheum.* **15,** 391 (1972).
14. J. P. CHANGEUX and J. C. GERHART, *Proc. 4th Meet. Fed. Eur. Biochem. Soc.* p. 13 (1967).
15. R. H. PERSELLIN and M. ZIFF, *Arthr. Rheum.* **9,** 57 (1966).
16. A. JANOFF, *Biochem. Pharmac.* **19,** 626 (1970).
17. J. D. JESSOP, *Br. Med. J.* **1,** 49 (1971).
18. F. E. KRUSIUS, A. MARKKANEN and P. PELTOLA, *Ann. rheum. Dis.* **29,** 232 (1970).
19. J. D. JESSOP, B. VERNON-ROBERTS and J. HARRIS, *Ann. rheum. Dis.* **31,** 535 (1972).
20. N. L. GOTTLIEB, P. M. SMITH and E. M. SMITH, *Arthr. Rheum.* **15,** 16 (1972).
21. D. METCALF, *Aust. J. Exp. Biol. Med. Sci.* **50,** 547 (1972).
22. P. A. CHERVENICK and A. F. LOBUGLIO, *Science* **178,** 164 (1972).
23. D. W. GOLDE, T. N. FINLEY and M. J. CLINE, *Lancet* **ii,** 1397 (1972).