CLINICAL AND EXPERIMENTAL STUDIES

WITH SODIUM AUROTHIOMALATE

by

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SUMMARY

Blood and urine gold levels were monitored in patients with rheumatoid arthritis treated with sodium aurothiomalate (Myochrysin). The results failed to demonstrate any association between the measured parameters and patient response. It was concluded that routine determinations of gold concentrations could not be used to predict which patients would fail to respond to treatment or would develop gold toxicity and the concept of therapeutic blood gold concentrations was shown to be invalid. The results were inconsistent with a previously proposed hypothesis that blood gold concentrations are inversely proportional to urinary gold excretion. Concentrations of gold in tissues removed from patients receiving Myochrysin showed that the metal was deposited in synovial tissue during chrysotherapy but that when treatment stopped, this gold was relocated elsewhere.

(1,4-\(^{14}\)C)thiomalic acid and sodium(1,4-\(^{14}\)C)aurothiomalate were prepared for experimental studies using rats. It was shown that a large proportion of the thiomalic acid remained at the site of intramuscular injection but sodium aurothiomalate was completely absorbed. Excretion and tissue retention of absorbed radioactivity showed that there was similar metabolism of the thiomalate moiety of both compounds suggesting a rapid removal of the gold from aurothiomalate. However, some differences in the urinary radioactive metabolites and tissue distribution pattern indicated that removal of gold was not complete and that some intact aurothiomalate remained. This inference was consistent with the results from \textit{in vitro} albumin binding experiments which demonstrated the presence of protein bound and non-bound aurothiomalate representing approximately 30\% of the initial material. Subcellular distribution and biliary excretion experiments showed that the gold and carbon-14 followed quite separate pathways intracellularly. Gold became concentrated in lysosomes whereas the major organelle which accumulated carbon-14 was the mitochondrion.

A wide range of interactions between gold and essential trace metals was demonstrated in a further series of experiments using rats. Accumulation of trace metals in the kidney was accompanied by the induction of a cytosolic gold-binding protein which also contained copper and zinc. Microscopic examination of renal tissue also showed that gold (and possibly copper) was also present within lysosomes of proximal tubular
cells. These animals also had evidence of renal tubular dysfunction. The results suggested that interactions between gold and trace metals, particularly copper, could be important in successful chrysotherapy and in gold toxicity. A mechanism for gold toxicity involving copper and metallothionein metabolism is proposed.
ACKNOWLEDGEMENTS

I am pleased to acknowledge the guidance, interest and consistently valuable advice throughout this study, shown by Dr. L.J. King. I would also like to acknowledge the special help given by Professor Vincent Marks, not only for the opportunities and facilities for the work to be carried out in his department, but also for his considerable and unceasing encouragement. To both Dr. King and Prof. Marks I record by sincere thanks and gratitude.

My thanks go also to Drs. R. Grahame and T. Gibson of Guys Hospital and to Drs. R. Hinton and B. Mullock at the Robens Institute, University of Surrey, for their tremendous help with much of the work.

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DEDICATION

To my wife and children for their love, understanding, tolerance and sacrifices.
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CHAPTER ONE

GENERAL INTRODUCTION
1.1 INTRODUCTION

Gold has captivated the interest of man for thousands of years. References to economic, decorative and artistic uses can be found in the oral, written and archeological histories of most ancient civilisations. From examples such as the treasures taken from the tomb of Tutankhamun and books of the Old Testament it is evident that gold has long had an important role in the ceremonies of many religious groups. From Greek literature, e.g. the story of Jason and the Golden Fleece, and from more recent history, e.g. the alchemists of the Middle Ages and the Spanish in Central America, it is seen that the desire to obtain the metal, powerfully influences attitudes and ambitions of men. These traditions can be followed through to the present day where frantic speculations seen on the world’s gold exchanges testify to the allure and power which is embodied within the metal.

In addition to this universal attraction to gold for its aesthetic properties and intrinsic value, a history of biological use exists which can be traced back to the Chinese civilisations of 2500 BC. (1,2)

1.2 BIOLOGICAL CHEMISTRY OF GOLD

The position of gold in the Periodic Table (Table 1.1) places the metal between the transition elements and the B-series of metals. Metallic gold, Au(0), is an extremely stable form but will react in aqueous solution with the thiol-containing compounds L-cysteine (I) and D-penicillamine (II) (3) Certain plants will accumulate gold from gold-rich soils and solubilisation of the metal in these situations may be effected by plant root micro-organisms able to secrete aminoacids. (4)

\[
\text{SHCH}_2\text{CHNH}_2\text{COOH} \quad \text{SH(CH}_3)_2\text{CCHNH}_2\text{COOH}
\]

L-cysteine (I) \quad \text{D-penicillamine(II)}

Table 1.1 POSITION AND FEATURES OF GOLD

Position in Periodic Table and electronic configurations of the metal and important ions.

<table>
<thead>
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<th>VIII</th>
<th>Group 1B</th>
<th>11B</th>
<th>Electronic Configuration</th>
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<tbody>
<tr>
<td>Ni</td>
<td>Cu</td>
<td>Zn</td>
<td>Au(0) 5s² 5p⁶ 5d¹⁰ 6s¹</td>
</tr>
<tr>
<td>Pd</td>
<td>Ag</td>
<td>Cd</td>
<td>Au(I) 5s² 5p⁶ 5d¹⁰</td>
</tr>
<tr>
<td>Pt</td>
<td>Au</td>
<td>Hg</td>
<td>Au(III) 5s² 5p⁶ 5d⁸</td>
</tr>
</tbody>
</table>
Of the higher oxidation states, compounds of Au(II) and Au(V) are very unstable in aqueous solution and are unlikely to be of biological importance although a potential role for Au(II) as an intermediate in redox reactions of Au(I) and Au(III) has been speculated upon. (4)

Reactions with Au(I) and Au(III) have been much more fully investigated. From the position in the Periodic Table and from the electronic configurations, it could be anticipated that gold would exhibit the properties both of the transition and the B-group metals. Au(I) would resemble the B-metals which have a closed d-shell and demonstrate covalent bonding while Au(III), with the unfilled d-shell, would form the ionic-ligand bonds typical of the transition metals.

1.2.1 Au(I), Aurous Compounds

The gold(I) cation does not exist in water, therefore while a wide range of aurous compounds have been formed, only complex compounds are stable in the presence of water. Gold(I) usually exhibits linear, twofold coordination due to the small energy difference between the filled 5d orbital and the unfilled 6s orbital which allows considerable hybridization of these orbitals. Examples of higher co-ordination numbers with trigonal planar and tetrahedral complexes have also been described. Soluble aurous complexes have been extensively investigated, particularly mercaptides, phosphines and cyanides which have greatest relevance to biological systems.

Gold(I)-thiol complexes: Au-S-R

Stable 1:1 thiol complexes readily form when gold(I) halide or cyanide is added to cysteine, penicillamine or glutathione(III) Analogous complexes will also form between cysteine or penicillamine and compounds of the type R$_2$PAuCl. Triethyl phosphine gold cysteine slowly breaks down to give gold(I) cysteine, whereas the triethyl phosphine gold penicillamine complex is stable to further substitution. (3) Paradoxically, the triphenyl phosphine gold complexes of cysteine and penicillamine are both stable.

\[
\text{COOH}_2\text{CHCH}_2\text{CH}_{2}\text{CONHCHCH}_2\text{SHCONHCH}_2\text{COOH}
\]

\[
\text{glutathione (III)}
\]
Penicillamine and AuCl$_2$ react to give Au(I) penicillamine in nitrogen or Au(III) penicillamine in air while cysteine forms only the Au(I) cysteine complex. (5)

These reactions suggest that in the presence of thiol ligands the Au(I) ⇌ Au(III) system may be very active and in vivo therefore, where several thiol compounds exist, gold(III) thiol complexes may be derived from an Au(I) precursor.

The structures of gold(I) complexes with other thiol compounds are less well defined. It has been suggested that sodium gold(I) thiomalate(IV) will form a stable species with thiomalic acid(V) until a gold:thiomalate ratio in excess of 1:1.75 is attained at which point free thiomalate is observed in a $^{13}$C nuclear magnetic resonance spectrum. (6) It is proposed that this stable complex has the formula $\text{Au}_4(\text{tm-S})_7^{3-}$ which, by analogy with observations on crystalline copper(I) thiols, could represent a tetrahedron of trigonally planar co-ordinated aurous ions located at the centres of opposite faces of ($\mu_2\text{tm-S})_6$ octahedron with one additional (fluxional) terminal tm-$\text{S}^-$. (6) The thiomalate ligand can be replaced from sodium gold thiomalate by strong sulphur donors such as cysteine. (7)

Reactions with the free thiol group on albumin have been investigated particularly with sodium gold thiomalate. The gold ion binds strongly to albumin-SH but the evidence for displacement of the thiomalate is contradictory. (7,8) Other thiol-binding species, e.g. drugs such as phenylbutazone, will in turn influence the reaction of albumin with gold.

Total or partial displacement of the ligand on the addition of other thiol compounds indicates the formation of mixed species. Isab and Sadler detected thiol exchange by $^{13}$C nuclear magnetic resonance when N-acetyl-L-cysteine, mercaptoacetic acid, L-cysteine methyl ester, aminoothane thiol, penicillamine or glutathione were added to sodium gold(I) thiomalate. (6) Sattari also prepared mixed thiol-gold complexes which he characterised by infra-red spectroscopy and elemental analysis. (9) Equimolar additions of cysteine to gold(I) thiomalate released half the thiomalate with the formation of cysteinatothiomalatodigold(I). Similarly,
glutathione displaced two thirds of the thiomalate and formed bis(glutathionato)mono(thiomalate)trigold(I). Sodium diethyldithiocarbamate however released all the thiomalate to give diethyldithiocarbamatogold(I). Sattari also noted that a thioether (methionine) did not react with gold(I) thiomalate.

Because of the widespread in vivo distribution of many of the compounds described in these experiments, it is probable that these gold thiol complexes and the mixed thiol-gold species will form and be of importance in the biochemistry of gold. Gold(I) thioglucose is extensively used in biological systems but the chemistry has not been examined as thoroughly as has that of gold(I) thiomalate.

Gold(I)-phosphine complexes: Au-P-R₃

Oral administration of gold(I) thiol compounds has proven to be unsuitable for achieving useful clinical or experimental levels in vivo. (10) Both absorption and excretion are rapid, and accumulation to effective concentrations therefore fails to occur. Slow release from a parenteral injection is the usual mode of presentation. Gold phosphine compounds however are usually soluble in organic solvents and will therefore interact with lipid media such as cell membranes. Thus gold phosphine complexes which do not require to be injected have recently been investigated as alternatives to gold thiol compounds. (11)

Bromo(triethylphosphine)gold reacts with a range of compounds but particularly with thiols, to form a mixed complex (Et₃PAuSR) by substitution of the bromide. Reaction with thiols occur readily in alkaline solution to form a monomeric product although, as noted above, some will slowly decompose to give triethylphosphine and an insoluble polymer of the gold thiol, e.g. gold(I) cysteine.

Triethylphosphine gold X (X = Cl or Br) compounds do not readily dissolve in water but do become soluble upon addition of sodium thiosulphate due to the formation of Et₃PAuS.SO₃. These compounds are readily oxidized with a further molecule of halogen to give the auric species Et₃PAuX₃. (12)

Triethylphosphine gold chloride is one gold phosphine compound which has been used in vivo but it is triethylphosphinegold-2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside that has been particularly investigated. (11)
Gold(l)-cyanide complexes

The most stable aurous complexes are those which involve the cyanide ligand. Gold(l) cyanide is soluble in cyanide solution to give the anion \((\text{Au(CN)}_2)^-\), a reaction which is exploited in the commercial extraction and recovery of gold. Fourfold co-ordination is observed when the potassium salt chelates with nitrogen ligands such as 2,2'-bipyridyl with the formation of complexes of the type \(\text{K(Au(CN)}_2\text{(bpy)})\). (12)

The use of aurous cyanide \(\text{Au(l)CN}\) as an \textit{in vitro} anti-bacterial agent marked the initiation of modern gold therapeutics. (13) It was the less than total success \textit{in vivo} and the accompanying toxicity which prompted a search for alternative gold drugs among the thiol and phosphine complexes.

While gold cyanide complexes were quite soon superseded as pharmacological compounds, most of the very few studies of gold(l) binding sites on proteins have involved \((\text{Au(CN)}_2)^-\). Gold inhibits the binding of \(\text{NAD}^+\) to the liver enzyme, alcohol dehydrogenase; \((\text{Au(CN)}_2)^-\) competes with the phosphate group on the co-enzyme thus preventing access to the binding site. (4) The gold does not appear to bind to available thiol groups on the enzyme, nor does it bind to the free-SH group of carbonic anhydrase. The link to this enzyme is thought to involve hydrogen bonding between a cyanide group and a zinc-co-ordinated water molecule held between histidine residues. (4)

The aurous complexes discussed are all stable in aqueous media. It is this property which has allowed them to be introduced into biological systems for a variety of purposes. Examples of reactions which may take place within such environments have been described and it is apparent that ligand exchange and the formation of mixed ligand clusters probably occur. Such exchanges may extend between the groups, e.g. phosphine-thiol, as well as within a group. Because of the extensive range of thiol compounds found within the body and their important biochemical roles, these molecules will be of considerable significance in the \textit{in vivo} fate of gold compounds.

1.2.2 \textbf{Au(III), Auric Compounds}

Gold(III) compounds are generally much more stable than corresponding aurous compounds. As with Au(l) there is one predominant co-ordination number, i.e. four, although a few compounds with five and six co-ordination have been described.
Compared with aurous compounds, auric compounds have been less extensively used in biological systems. Often it has been assumed that a similar biochemistry would obtain and the application of neutral solution of auric chloride was once proposed as an antiseptic eye-wash. (14) This assumption however is unlikely to be valid; auric compounds are strong oxidising agents and may be rapidly reduced to aurous gold to which any subsequent reaction would be attributed. However, stability is increased by the presence of nitrogen ligands and therefore some Au(III) compounds within biological systems (which are normally mildly reducing) need not necessarily go to Au(1).

As with gold(I), an extensive range of compounds have been prepared but very few are of relevance to the biological chemistry of gold(III). (12) Both cysteine and glutathione will reduce gold(III) compounds to form the appropriate aurous thiolate. Likewise gold(III) thiomalate will not stabilise in aqueous solution but reduces to the gold(I) complex. With 2,3-dimercaptopropanol (British Anti-Lewisite, BAL)(VI), a range of insoluble compounds, indicative of Au(I) and Au(III) polymeric species are formed in association with \((\text{AuCl}_4)^-\). By contrast, gold(I) penicillamine formed under aerobic conditions readily oxidises to produce the gold(III) penicillamine complex.

\[
\text{CH}_2\text{SHCHSHCH}_2\text{OH (VI)}
\]

Tetrachloraurate will cause precipitation of albumin if present in sufficient concentration while at lower concentrations reduction of the gold(III) takes place.

Since there is some stability in biological systems of gold(III) complexes and since the auric species may even be favoured in some situations, it is important to consider possible ligand exchange. Such reactions have not been well studied in aqueous media but from the data available it is evident that exchange on Au(III) is very slow in comparison with Au(I). Hydrolysis of \((\text{AuCl}_4)^-\) proceeds stepwise \(\rightarrow \text{AuOHCl}_3^- \rightarrow \text{Au(OH)}_2\text{Cl}_2^- \rightarrow \text{Au(OH)}_3\text{Cl}^- \rightarrow \text{Au(OH)}_4^-\). The first hydrolysis is complete within 1 minute, the second within 1 hour while the last two require several hours. (15) Other examples include substitutions which readily occur with aurous compounds but fail to take place at all with the corresponding auric complex. (4) These examples illustrate the greater strength of an Au(III)-X bond compared with Au(I)-X.
1.2.3 Au(III), Protein Labelling

In the determination of protein structure by X-ray crystallography, gold has been used as a marker in order to label and identify areas of the molecules. Thus there are considerable data available on gold binding sites for several proteins including Bence-Jones protein, (16) chymotrypsin, (17) yeast phosphoglycerate kinase, (18) and lactate dehydrogenase. (19) The nature of the binding is very dependent upon the experimental conditions. With excess Cl⁻, (AuCl₄⁻) binds to positively charged arginine residues on lysozyme, (20) while with sperm whale myoglobin in an ammonia buffer, complexes such as Au(NH₃)₄³⁺ and Au(NH₃)₃NH₂⁺²⁺ are presumed to form, which then bind to carbonyl groups. (21) As with Au(I), binding to the thiol groups of cysteine residues is a possibility but reduction to aurous gold is likely to occur.

Insoluble complexes are formed between (AuCl₄⁻) and purines such as adenine. (22) Tetrachloraurate and adenosine nucleotide however produces a soluble complex. (23) Although ribose, deoxyribose-5-phosphate, sucrose or dextrose all promote the reduction of Au(III) to Au(0) this is not achieved by the nucleotide. Pyrimidine complexes with gold(III) have also been prepared and gold 5-diazouracil, (Au(5du)₂Cl₂)Cl.HCl has anti-tumour activity in mice. (24)

Therefore, from the chemistry of auric compounds it would be anticipated that in most situations Au(III) introduced into a physiological milieu would soon be reduced to aurous gold or even to the metal. However, the stability of a few compounds such as gold(III) penicillamine suggest that where appropriate conditions exist, auric compounds may be localised or even be formed. Where present these compounds are stable to substitution or exchange. However, it is the gold(I) complexes which in general will probably be of major biological importance.

1.2.4 Compounds with Gold-Metal Bonds

A number of compounds containing gold-metal bonds have recently been prepared and can be classified into two groups:

(a) Compounds in which several gold atoms are clustered in discrete organisations. The (Au₄(tm-s)₇)³⁻ species referred to above is an example although clusters containing as many as eleven gold atoms have been described with even larger organisations predicted. (25)
(b) Compounds containing direct Au-M bonds. Simple dimers, e.g. Au-Au, Au-Ag, Au-Cu are observed in gaseous phases but complexes of the type LAuMn(CO)_5 or LAuMn(CO)_4L are much more common. In these prepared compounds, L, and L are usually triphenylphosphine or a simple derivative. The reactions of these compounds are similar to those of carbonyl halides, Mn(CO)_5X, suggesting that the LAu group behaves in an analogous fashion, to a pseudo halogen. Whether such compounds could form in vivo has not been suggested.

1.2.5 Summary

Of the various gold oxidation states, Au(I) and Au(III) are essentially the only two which could exist within an aqueous physiological environment. While in general terms Au(III) compounds are more stable than those of Au(I), it is the aurous complexes which are most likely to exist in biological systems, the auric gold being reduced to aurous. By virtue of their natural abundance, thiol complexes with gold(I) are the most important, although phosphine and cyanide compounds, when introduced, can be expected to react with physiological ligands such as protein, to induce biochemical effects. Gold(III) ligand bonds are very stable and these complexes therefore probably give rise to less dramatic responses.

Substitution and exchange of ligands associated with aurous complexes have been shown to occur very readily in vitro, and it is likely that similar reactions will occur in vivo where a variety of intra- and extracellular ligands exist. Gold clusters which have been demonstrated in aqueous solution may also form in vivo, although these have not been sought. The possible existence of other direct gold-metal interactions has similarly so far been overlooked.

1.3 CHEMOTHERAPEUTICS OF GOLD

Gold, and gold compounds have retained a favoured place in the pharmacopea of "physicians" since at least the beginnings of recorded history. A formulation of auric chloride, prepared by dissolving gold in aqua regia and neutralised with chalk, was recommended by Roger Bacon in the thirteenth century as a treatment for leprosy. At various times since, gold therapy has been claimed to be a "miracle cure", effective for virtually every disorder. However, since large numbers of the populations continued to suffer with painful disease and to be vulnerable to infection, presumably the treatment was not available other than to an influential minority or
else such assertions were not taken entirely seriously except perhaps by
the proponents who no doubt had a commercial interest in their acceptance.
During the last century gold compounds have been used on a more rational
and scientific basis with particular emphasis in recent years upon
conditions with an auto-immune element. (Fig. 1.1)

1.3.1 Gold Therapy; Antibacterial Activity

Antibacterial effects of gold were first demonstrated ninety years ago. (13)
Koch showed in 1890 that potassium auricyanide inhibits the growth of
*Mycobacterium tuberculosis* when added to culture medium at a concentration
of one part in two million. This was followed by a report that intravenously
administered sodium aurichloride had antibacterial effects in experimental
tuberculosis. (26) The associated toxicity prohibited immediate thoughts
of clinical use. However, by the second decade of the twentieth century
attempts were being made and despite the hazards, compounds such as
potassium auricyanide were reported to have therapeutic activity in cases
of tuberculosis and also in syphilis. (27)

The encouraging response prompted the development of more than twenty new
gold complexes in a search for compounds which would provide efficacy but
have lower toxicity. An immediate example, reported in 1914, was 'Auro-
cantan'. (Table 1.2) This compound was given by sub-cutaneous injection
but was of limited value causing tissue necrosis after repeated admin-
istration. (10)

Those compounds which demonstrated favourable anti-tubercular and anti-
syphilitic activity were rapidly introduced for the treatment of disease
with minimal pre-clinical and clinical trials. Organo-gold complexes were
first introduced at this stage with the synthesis of 2-aurothio-4-amino-
benzoic acid ('Krysolgan'). (28) It was claimed that this compound was as
effective as the toxic inorganic gold compounds. Other workers confirmed
this view but found it to be equally as toxic also. (10) Several other
gold-sulphur complexes and one gold-nitrogen complex were prepared during
this period and were all administered intravenously. (Table 1.2)

Sodium aurothiosulphate, first investigated in 1913 received considerable
interest and was introduced for the treatment, by intravenous injection,
of tuberculosis in 1924. (29) It remained the drug of choice until anti-
tuberculosis therapy entered a new phase with the development of the
modern drugs isoniazid, para-aminosalicylic acid and rifampicin.
FIGURE 1.1 LANDMARKS IN GOLD CHEMOTHERAPY

Bronchial Asthma

Pemphigus

Rheumatoid Arthritis

Antibacterial Agents

Leprosy

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<thead>
<tr>
<th>Auric Chloride</th>
<th>Gold Sulphate</th>
<th>Aurothiomalate</th>
<th>Aurothiobenzoic Acid</th>
<th>Aurothioglucose</th>
<th>Allochrysine</th>
<th>Triethyl Phosphine</th>
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<td>Cantharidylethylenediamine aurocyanide (Aurocantan)</td>
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<td>Sodium aurothiosulphate (Sanochrysin)</td>
<td>$\text{Na}_3\text{Au}(\text{S}_2\text{O}_3)_2$</td>
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<td>2-aurothio-4-amino- benzoic acid (Krysolgan)</td>
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<td>Gold keratinate (Auro-Detoxin)</td>
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<td>Sodium 3-aurothio-2-propanol-1-sulphonate (Allochrysin)</td>
<td>$\text{Au-S-CH}_2\text{CHOH-CH}_2\text{SO}_3\text{Na}^+$</td>
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<td>Aurothioglucose (Solganal B Oleosum)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Sodium aurothiomalate (Myochrysin)</td>
<td>$\text{CH}_2\text{COO Na}$ $\text{AuS-CH-COO Na}$</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>3-triethylphosphinegold(I)-2,3,4,6-tetra-O-acetyl-l-thio-β-D-glucopyranoside (Auranofin (SK and F D-39162))</td>
<td>$\text{Et}_3\text{P-Au-S}$ $\text{R = CH}_3\text{CO}_2$</td>
<td></td>
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</tbody>
</table>
1.3.2 Gold Therapy; Rheumatoid Arthritis - Introduction

While these gold compounds were being developed for the treatment of diseases associated with micro-organisms they were not used exclusively for such purposes. Fifty to sixty years ago it was (erroneously) believed that what are now recognised to be completely separate conditions, were manifestations of tuberculosis. Therefore, patients with lupus erythematosus (LE) were treated with various gold compounds by Martenstein. (30) With a similar rationale, Forestier (31) adopted gold therapy (chrysotherapy) for rheumatoid arthritis using the newly developed gold compound sodium 3-aurothio-2-propanol-1'sulphonate ('Allochrysin') and later, sodium aurothicmalate ('Myochrysin'). (Table 1.2) Lack of success in the first disorder led to the immediate demise of gold treatment in LE. However, the considerable success achieved with what was hitherto a completely intractable disease, quickly established gold therapy as a valuable weapon against rheumatoid arthritis.

Chrysotherapy for rheumatoid arthritis, however, was no less free from the problems of associated toxicity than in tuberculosis and in efforts to minimise the side-effects further gold preparations (Table 1.2) continued to be introduced. Experience during the succeeding years confirmed sodium aurothiomalate and aurothioglucone as the two compounds which were most effective and had an acceptable level of toxicity so that by 1950 no other preparations were in routine use. Both were administered by intra-muscular injection.

Forestier's initial observations concerning the effects of gold on rheumatoid arthritis were substantiated in many other reports (32-34) but some doubts on the value of chrysotherapy were later expressed when it was pointed out that with one exception (35) studies had omitted to include control series of patients. There arose therefore considerable doubt and confusion surrounding a treatment which had been in use for thirty years. Such a situation could not be allowed to continue and the Empire Rheumatism Council (ERC) organised a multi-centre, double-blind controlled trial to determine; Does gold have a beneficial action on rheumatoid arthritis?

Patients with active rheumatoid arthritis of 1 to 5 years' duration were included in the trial. The test group received 20 injections of 50 mg sodium aurothiomalate (total dose - 1.0 g) and the control group received 20 injections containing 0.5 μg (total dose - 0.01 g). The group treated
with gold showed improvement of their disease when judged by clinical and laboratory criteria, which was greater than that observed in the controls. (36,37) The question was therefore answered and the drug continues still to be widely used. Since the final report of the ERC multi-centre trial the one significant development in gold therapy for rheumatoid arthritis has been the introduction of a gold complex which can be administered orally. This has the advantages of reducing patient discomfort and easing administration.

Following trials in adjuvant arthritic rats, initially with triethylphosphinegold chloride and subsequently with other trialkylphosphinegold complexes, 3-triethylphosphinegold - 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside (SK & F D-39162, 'Auranofin') was used to treat patients with rheumatoid arthritis. (11) The first report appeared in 1976 and in 8 patients who were treated and studied for only six months, improvement of the disease was confirmed by clinical and laboratory assessment. Reports of larger and longer trials, including control series of patients, and documentation of side-effects are still awaited but the initial evidence is that oral chrysotherapy is as effective as the conventional treatment.

Therefore, chrysotherapy for the treatment of refractory rheumatoid arthritis has a well established role which shows no sign of being displaced by alternative drugs of more recent development. Gold therapy for tuberculosis has been abandoned but there are a number of other conditions in which gold or gold compounds are used. Such cases are fewer than those of rheumatoid arthritis but nonetheless they help to emphasise the considerable value of chrysotherapy.

1.3.3 Gold Therapy: Non-Rheumatoid Arthritis

Psoriatic Arthritis

Psoriasis is a fluctuating disease which affects the skin and nails. A dry, scaling rash is found, often on extensor surfaces but sometimes also on the scalp, in the axillae, under the breasts or in the genito-anal region. The nails have a characteristic "thimble pitting" and, in severe forms, subungual separation. (38) In about 10% of patients a seronegative, anodular, erosive arthritis occurs, generally in association with a relapse of the dermatological symptoms. In a small proportion of patients, however, the arthritis can develop before the eventual appearance of skin lesions.

Chrysotherapy for psoriatic arthritis was first suggested in 1959 and sporadic reports since then have advocated its use. (39) From a large
A retrospective study of the treatments employed in 98 patients with the disease it was concluded that chrysotherapy has a definite place in the management of severe psoriatic arthropathy and that the incidence of severe toxic reaction is low. (40)

Sodium aurothiomalate is no more toxic in children than in adults and chrysotherapy is therefore appropriate in Still's Disease (Juvenile Chronic Polyarthritis). (41) Gold treatment has also been used in a limited series of patients with Palindromic arthritis.

Other conditions which have been successfully treated with sodium aurothiomalate also have an immunological involvement. Pemphigus is a chronic disease in which patients have intra-epidermal acantholytic bullae and produce an anti-epithelial antibody. A high mortality rate is found in untreated disease and prolonged use of systemic corticosteroids causes undesirable morbidity. Chrysotherapy was introduced in 1973 and subsequent experience suggests that with careful supervision a good response rate with a very low incidence of gold toxicity can be achieved. (42) There are also reports from Japan of chrysotherapy used to treat bronchial asthma. (43)

1.3.4 Gold Therapy; Miscellaneous Uses

Extensive joint inflammation and effusion which is unresponsive to the usual methods of control may be treated by surgical removal of synovial tissue. As an alternative, synovectomy can be accomplished by intra-articular injection of radioactive $^{198}$Au colloidal gold. (44) $^{198}$Au colloidal gold has also been used as an in situ cancer chemotherapeutic agent by instillation into body cavities where it limits spread of metastatic disease. (45)

As with gold salts a history of medical use of metallic gold can be traced over many centuries, principally in dentistry. 

1.4 RHEUMATOID ARTHRITIS

1.4.1 Introduction

It has been seen that gold has been and continues to be applied to a wide spectrum of clinical problems ranging from those associated with experimental physiology to the control of various unrelated diseases.
Despite being employed for these multi-various purposes it is the treatment of rheumatoid arthritis which presents the primary target upon which chrysotherapy is concentrated. Work described in later chapters concerns studies in patients with rheumatoid arthritis treated with gold - which also provided the stimulus for the experimental investigations included. Thus it is relevant to provide a brief description of this disease.

Rheumatoid arthritis is an inflammatory disease of synovial tissue which leads to destructive changes in the underlying joints. Arthritis (joint inflammation) is the most obvious and frequent manifestation but many other systems may also be involved in what is essentially a generalised disease. Abnormal antibodies (rheumatoid factors) are present in the blood of affected subjects. It is an extremely painful and crippling disease for which the cause remains unknown and which can be very difficult to control and treat. Lansbury has written that rheumatoid arthritis "may be likened to a smouldering fire which slowly consumes the body's supporting structures and ultimately leaves behind a mass of twisted wreckage". (46)

1.4.2 Clinical and Pathological Features

The fundamental feature of rheumatoid arthritis is synovitis involving the joints, bursa and tendon sheaths. The synovium becomes oedematous with infiltration by lymphocytes and plasma cells aggregated into follicles. Erosions to both the cartilage and underlying bone at the cartilage/synovial junction occur to leave large cystic areas which can be seen radiologically within a year of onset. (47)

Rheumatoid arthritis has many systemic features in addition to those associated with the inflammation and damage to joints and bone. Subcutaneous nodules are one such feature which is almost pathognomonic of rheumatoid arthritis (occasionally observed also in systemic lupus erythematosus). The nodules are small masses, 2 mm - 3 cm in diameter, located at pressure points such as the elbows and Achilles tendon and sometimes over joints of the fingers and toes. They are found in 25% of patients with rheumatoid arthritis, are non-tender or painful and histologically form a non-encapsulated zonal granuloma.

Arteritis is an important development in the disease. Inflammation causing arterial occlusion occurs only in those patients who are strongly sero-positive and usually also have subcutaneous nodules.
Patients with rheumatoid arthritis have a higher incidence of previous chest disease than the general population but may also suffer from various lesions to which no cause can be attributed and are therefore collectively described as rheumatoid lung. These lesions include:

- pleural effusion; sterile pleural fluid produced in association with chronic inflammatory changes in pleural biopsy tissue. Occurs in a small proportion of male patients.

- fibrosing alveolitis; impaired pulmonary function.

- rheumatoid nodules - of the same histological appearance as subcutaneous nodules but found in lung parenchymal tissue.

- pneumoconiosis; a radiological picture of reticular shadowing and peripheral opacities.

Rheumatoid arthritis is invariably accompanied by an anaemia due to iron and folic acid deficiency. Anaemia may also form part of Felty's Syndrome which is the combination of rheumatoid arthritis, splenomegaly and neutropenia. Associated features include low resistance to infection, weight loss, lymph node enlargement, skin pigmentation and leg ulceration.

The natural history of the disease does not follow a predictable pattern but develops along a course typical only to the individual. The course may be punctuated by spontaneous improvement or exacerbation and permanent remission may occur at any stage. In only a small number of patients does the disease produce extensive disability and an inevitable progression to overwhelming crippling is not the usual prospect.

The signs which are predictive of a poor prognosis include: insidious onset of the disease, failure to go into remission during the first year, early appearance of radiological erosions and of nodules, high levels and early appearance of the laboratory monitors of disease activity. As active progressive disease continues for longer periods, the final outcome can be predicted to be proportionately more severe.

Three times as many women are affected with rheumatoid arthritis as are men. The disease may develop at any age although numbers increase with a
peak age of onset during the fifth decade. Within the aged population (over 65 years) the incidence of the disease is three times greater than within the general population.

From studies with families it appears probable that a small genetic component to the disease exists. The incidence of rheumatoid arthritis and the prevalence of serum rheumatoid factors are slightly greater in close relatives than in controls. However, any genetic influence which operates is very weak in comparison with other components. (48)

Rheumatoid arthritis affects populations in all parts of the world irrespective of climatic conditions or ethnic origins. The incidence in underdeveloped countries is lower than is found in the more advanced countries but this probably reflects the reduced life expectancy of the population, especially the women.

1.4.3 Aetiology of Rheumatoid Arthritis

The aetiology of rheumatoid arthritis remains one of the greatest enigmas in modern medicine. Despite the considerable research extending for more than fifty years and the embarrassing excess of clinical material for study, the origins of the disease are as yet unknown. In the context of a discussion on dermatology, Shuster remarked "facts are as common as beard hairs and by themselves are as useless". He could, just as appropriately, have been thinking of what we know of rheumatoid arthritis where observations are legion and almost overwhelm attempts to define the cause of the disease.

Despite this perhaps pessimistic introduction, hypotheses have been assembled into which much of the evidence can be slotted to give reasonable working models, albeit ones that are incomplete. Although the cause is obscure, it is believed that development of the disease begins with an initiating factor which sets up an immune reaction, and also involves one or more perpetuating factors.

A number of initiating factors have been proposed but the participation of any of them has never been clearly demonstrated. The possible role of an infective agent is suggested by the evidence that rheumatoid arthritis is a comparatively recent disease of mankind. At the same time (a) Arthritis is frequently associated with viral infections such as rubella
(although the arthritis rarely persists); (b) Serum rheumatoid factors (see below) are found during many infective illnesses (see Table 1.3) and in animals treated with repeated injection of killed bacteria; (c) Many of the systemic features of rheumatoid arthritis, e.g. fever, lymphadenopathy and splenomegaly, are similar to those of infection and in many patients the onset of their illness is accompanied by an infection of the upper respiratory tract; (d) Rheumatoid-like diseases can be produced in animals by injection with micro-organisms.

Despite this suggestive data that an infection may precipitate the disease, it has not proved possible to isolate a causative agent from an affected joint. Warren et al. prepared a saline extract of synovial tissue from patients with rheumatoid arthritis. (49) The offspring of mice injected with the extract developed arthritis as did the next generation, suggesting that some transmissible agent was present. Mycoplasma fermentans, diphtheroids and Listeria have reportedly been isolated from rheumatoid joints but attempts to isolate viruses have been unsuccessful. (50-52)

Gerber has proposed an alternative hypothesis which is consistent with many of the biochemical changes observed in the serum and synovial fluid of patients with rheumatoid arthritis. (53) He suggests that abnormal aggregations of gammaglobulin within the synovial fluid precipitates an antigenic reaction with the appearance of rheumatoid factors and joint inflammation. The argument upon which the hypothesis (which is shown in Fig. 1.2) is developed, is as follows:--

<table>
<thead>
<tr>
<th>Table 1.3 CAUSES OF POSITIVE RHEUMATOID FACTOR TESTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONDITION</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>Normal persons</td>
</tr>
<tr>
<td>Relatives of rheumatoid arthritis patients</td>
</tr>
<tr>
<td>SLE</td>
</tr>
<tr>
<td>Progressive systemic sclerosis</td>
</tr>
<tr>
<td>Subacute bacterial endocarditis</td>
</tr>
</tbody>
</table>
Table 1.3 (contd)

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver diseases</td>
<td>Especially infective hepatitis, chronic active hepatitis and cirrhosis.</td>
</tr>
<tr>
<td>Pulmonary fibrosis</td>
<td>Not necessarily associated with rheumatoid arthritis.</td>
</tr>
<tr>
<td>Leprosy</td>
<td>Up to 50%</td>
</tr>
<tr>
<td>Chronic pulmonary tuberculosis</td>
<td>5-20%</td>
</tr>
<tr>
<td>Conditions with raised e.g. sarcoidosis, multiple myeloma, gammaglobulin levels</td>
<td>macroglobulinaemia.</td>
</tr>
<tr>
<td>Sicca syndrome</td>
<td>Almost 100%</td>
</tr>
</tbody>
</table>

Protein aggregation \textit{in vitro} is promoted by copper and by hyaluronic acid but is inhibited by histidine. (53) The \textit{in vitro} depolymerization of hyaluronic acid by ascorbic acid is inhibited by caeruloplasmin. (54) Abnormal aggregates of immunoglobulin are found in the synovial fluid of patients with rheumatoid arthritis. (55) Also in patients with rheumatoid arthritis the concentration of thiol groups (e.g. protein) in serum is reduced while the concentrations of copper and caeruloplasmin in both serum and synovial fluid are increased. (56-58) The serum free histidine concentration is decreased, (59) and there are increased amounts of hyaluronic acid at the joint (the concentration is reduced but fluid volume is much greater). (60) Thus the conditions which are favourable for protein aggregation may well exist in the synovial fluid of these patients.

Further evidence to support the hypothesis is found from:

1. experiments where copper was added to plasma and abnormal, heavy, sedimenting protein fractions were observed on ultracentrifugation (53),

2. the urinary excretion of a copper-ligand was determined in rheumatoid arthritis patients. The ligand was not positively characterised but it was not a sulphur donor and similar behaviour was noted with histidine and 3-methyl-histidine. (61)
Fig. 1.2 Biochemical Changes Associated with Rheumatoid Arthritis

The features shown are of possible importance in the development of the disease and the inflammatory response at the joint.

<table>
<thead>
<tr>
<th>URINE</th>
<th>PLASMA</th>
<th>SYNOVIAL FLUID</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>↓-SH</td>
<td>ascorbic acid</td>
</tr>
<tr>
<td></td>
<td>↑caeruloplasmin</td>
<td>hyaluronic acid ↑</td>
</tr>
<tr>
<td>copper - (histidine) complex</td>
<td>↑copper</td>
<td>caeruloplasmin ↑</td>
</tr>
<tr>
<td>histidine</td>
<td>↓</td>
<td>copper</td>
</tr>
<tr>
<td>Rheumatoid factors</td>
<td>Immune complexes</td>
<td>Rheumatoid factors</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SYNOVIAL MEMBRANE</td>
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</tbody>
</table>

INFLAMMATION
These data implicate copper and its carrier protein caeruloplasmin at several sites in the hypothesis with the inference that copper metabolism is particularly important in the aetiology of rheumatoid arthritis.

Myoehrisin and penicillamine are reported to inhibit protein aggregation. (53) The hypothesis would predict an easing of the antigenic reaction with a subsequent decline in the production of rheumatoid factors and subsidence of the inflammation. This is the response which is observed in the majority of patients treated with these drugs.

The biochemical hypothesis offers no explanation of the disorder in copper metabolism and there is no attempt to apply the theory to the systemic symptoms. Less attention has been paid than has been given to the proposal of an infective agent as initiator.

Transition from the initial response to the chronic disease with inflammation and joint destruction is marked by increased immune activity. According to the supposed initiating factor, this overactivity may be driven on by persistent exposure to the infective organism or the continuing alteration in protein structure. This immunological activity may become self-perpetuating as an auto-immune disease is generated in response to a change in the antigenic specificity of some component within the joint.

The immunological features of the disease are revealed by the presence of plasma cells and lymphocytes within the joint, the formation of rheumatoid factors and immune complexes with their appearance in serum and reduced complement activity in synovial fluid.

Rheumatoid factors are antibodies directed against antigenic determinants on the Fc fragment of the gammaglobulin, IgG. In most cases the antibody is of the type IgM, and it is this immunoglobulin which is determined in conventional laboratory tests (see Section 1.4.4). The presence of IgM rheumatoid factors defines a patient as having seropositive disease. More recently it has been shown that some patients will have IgG or IgA type rheumatoid factors. Rheumatoid factors circulate as soluble complexes, one molecule of IgM combined with five or six molecules of IgG. Rheumatoid factor in the serum of a patient represents a series of different antibodies, each of which will react specifically with a different mammalian IgG.
The rheumatoid factors are produced in lymph nodes and locally in the synovium by plasma cells. Since rheumatoid factors are found in the serum of some healthy persons and of patients with other diseases, and since animals immunised with their own IgG (in Freund's adjuvant) will produce rheumatoid factor but do not develop arthritis, it is probable that rheumatoid factors do not themselves cause the rheumatoid arthritis but are associated with it. (62) The immune complexes however may be more involved with the pathological lesions. They are believed to activate complement and become phagocytosed by granulocytes in the synovial fluid. This process may precipitate a release of lysosomal enzymes from the granulocyte which, with the activated complement, may then stimulate an inflammatory response on the synovium. Fluid from inflamed joints is known to contain lysosomal enzymes and has a reduced total haemolytic complement level. (63)

Despite this rather simplified description it is evident that a major feature of rheumatoid arthritis is the activity of the immune mechanism and response. There is good evidence to indicate that a considerable portion of this activity is of an auto-immune nature and that rheumatoid factors and immune complexes at least reflect disease activity and are probably involved in stimulating inflammation. None of the theories adequately explain all the changes in biochemistry and serology which are detected in patients with this disease.

1.4.4 Special Investigations

A considerable number of laboratory and other investigative procedures have been described which may be informative in the differential diagnosis of connective tissue disorders and in establishing the severity of the disease. Some of these tests are regularly repeated in order to follow the development of the condition and to assess the response to treatment. There is a second group of investigations in which abnormal results have been found in patients with rheumatoid arthritis but the significance of which is uncertain. These tests have not found a place in the routine assessment of patients although most have ardent proponents who regularly proclaim their virtue.

Routine Investigations

1. Radiology. Early radiological signs reflect only the inflammation, showing soft shadows around swollen joints, osteoporosis and narrow-
ing of joint space. These changes are typically observed at the small joints of the hands and feet although other joints e.g. the hip are subsequently affected in a similar way. With the progression of the disease more pronounced erosive changes are seen. Collapse and mal-alignment of joints may be the eventual outcome of bone erosion which is seen but other, stable deformities can be produced.

2. Erythrocyte Sedimentation Rate (ESR). The sedimentation rate reflects inflammatory activity and is an indispensible investigation. While the inter-individual variation is considerable, repeated measurements on the same person provide an extremely useful guide to the state of the disease.

3. Tests for Rheumatoid Factors. Rheumatoid factor is detected by its effect upon an IgG (human or rabbit)/particle complex. In the presence of rheumatoid factor these particles will agglutinate to give a visible flocculate or precipitate. Two basic procedures have been described for measurements of rheumatoid factor - (a) Latex flocculation and (b) Rose-Waaler (Sheep cell) tests. The latter test has been refined into the sheep cell agglutination test (SCAT) and the differential agglutination test (DAT).

The two tests are often used to complement each other. Because human IgG is present in the latex test, this is a more sensitive procedure but has poor specificity for rheumatoid arthritis. The sheep cell methods are more elaborate to perform but are less subject to interference. The former is therefore a convenient, rapid screening test with the latter used for confirmation of positive results.

4. Haematological Status. Because of the anaemia consequent upon the chronic inflammatory nature of the disease it is important to monitor iron and folate status of the patients. Routine haematological tests are therefore undertaken at the same time as the erythrocyte sedimentation rate is measured.

Non-Routine Investigations

There have been a limited number of studies to assess the effect of rheumatoid disease upon the chemical composition of serum. The systemic disease affects several organs and it would be expected therefore that serum chemistry would be altered in response to this involvement.
Wilding et al. measured thirteen parameters of serum chemistry, ESR, haemoglobin and white cell count, in patients with arthritis in order to determine which of the tests routinely performed in hospital laboratories best represent the biochemical and haematological profile of rheumatoid arthritis. (64) More than half the biochemical and all three haematological tests were significantly different in the patients with rheumatoid arthritis compared with the reference population. Albumin and bilirubin showed greatest shifts away from the reference values. The most important finding from this and similar studies was the demonstration of a reduced concentration of serum albumin which was found to be as good as the ESR as an index of rheumatoid inflammatory activity. Impaired hepatic synthesis may be responsible for this (cholesterol concentrations were also reduced) but there are studies which suggest that in rheumatoid arthritis the rate of catabolism is increased. (65)

McConkey et al. have argued that as a guide to the eventual structural damage wrought by the disease, the intensity and duration of inflammatory activity serves as a good index. (66) In addition to the ESR they demonstrated that acute phase reactants (the proteins produced in response to a non-specific tissue injury and inflammation), measured in the serum, are reliable markers of control over inflammatory and rheumatoid disease activity.

The biochemical hypothesis for the aetiology of rheumatoid arthritis was elaborated by Gerber (see Section 1.4.3) to include observed changes in the concentrations of components in the synovial fluid. Measurements of copper, caeruloplasmin and histidine etc. which have been made in synovial fluid from patients and controls have shown that concentrations of these elements in the synovial fluid of subjects with rheumatoid arthritis are consistent with the hypothesis. Collection of synovial fluid however is not a procedure which is readily carried out and therefore measurements of the serum concentrations have been performed to provide an assessment of fluid content.

Many reports have shown that in patients with rheumatoid arthritis the concentration of free histidine in serum is significantly decreased compared with the histidinaemia of normal subjects and patients with non-rheumatoid diseases. (67,68) This hypohistidinaemia was profound in both men and women and correlated with the severity of disease as demonstrated by a range of parameters. (59)
The serum concentration of copper and its carrier protein caeruloplasmin were shown to be increased in patients with rheumatoid arthritis as long ago as 1958 and there have been many subsequent reports confirming this observation. (57,58,69,70,71) The increased serum copper concentration could be a non-specific effect as caeruloplasmin (an acute phase reactant) is released in response to the disease. However, several studies have demonstrated an increase in non-caeruloplasmin bound copper suggesting that additional mechanisms are operating. (58,71) At the same time as the serum copper increases the concentration of zinc falls. (72) Therefore the copper/zinc ratio provides a further parameter which can be used to assess disease activity together with ESR, rheumatoid factor, acute phase reactants and histidine. (73)

Examination of synovial fluid. Synovial fluid may occasionally be aspirated from an inflamed joint to relieve pressure or to assist with a diagnosis when a patient is first seen. Unlike the viscous, straw-coloured synovial fluid from a normal joint the aspirate from an inflamed joint is opaque, less viscous and of a much greater volume. A grey, turbid fluid is indicative of septic arthritis. Fluid can be examined for cells and micro-organisms and if the diagnosis is uncertain, measurement of rheumatoid factor may be helpful.

Other investigations are possible but are usually of academic rather than clinical relevance. Thus, as has been noted earlier, copper, caeruloplasmin, hyaluronic acid and histidine concentrations have been measured in fluid and the activities of various enzymes also measured, particularly the hydrolases released from lysozomes.

1.4.5 Treatment

Treatment of rheumatoid arthritis is multi-faceted and affords an example of the value of multidisciplinary teams focussing a range of medical expertise upon the problems presented by a patient with a serious disease.

For the patient with rheumatoid arthritis the following therapeutic and social options are available, although not all may necessarily have to be used; rest, provision of supportive appliances, physiotherapy, occupational therapy, provision of appropriate housing aids etc., drugs and surgery. While all are important in the management of the patient, consideration in this discussion will be given only to drug therapy.
Rheumatoid arthritis causes a patient a number of problems which can be treated with drugs. These include pain and the associated difficulty with sleeping, depression, inflammation and joint stiffness, dysfunction to associated systemic organs and the active, erosive disease itself. The simple classification of treatment with drugs recognises (a) non-specific or symptomatic therapy and (b) specific therapy which acts slowly upon the rheumatic disease process to alter the course of a progressive disease. Included in the first group are the so-called "first-line drugs" which are analgesics, and the "second-line drugs" which are represented by non-steroidal anti-inflammatory drugs (NSAID's). The specific agents form the "third-line drugs", and very occasionally it may also be necessary to make limited use of steroids either locally or systemically. Thus a stepwise pharmacological approach to the disease is evident, the patient initially receiving pain relief and as necessary progressing to more aggressive forms of treatment.

Analgesics

Not all patients will require any other treatment than simple pain relief. Aspirin is the traditional drug of choice, but cannot be used without a certain risk. (41) In very severe disease where pain is considered unbearable and the prognosis very poor, addictive analgesics such as morphine may be prescribed.

Non-Steroidal Anti-Inflammatory Drugs

By reducing swelling and pain, and by allowing improved joint function, these drugs considerably help the clinical condition. They represent the major form of drug treatment for rheumatoid arthritis, and a large number of such compounds are now available.

Specific Drugs

These agents must be taken for several weeks before evidence of improvement becomes apparent. If therapy is ceased prematurely the subsequent relapse is also delayed. Patients for whom specific therapy is appropriate are those with acute inflammatory rheumatoid changes with active and progressive disease and in whom NSAID's have proved ineffective. Their introduction should not be considered unless the disease has been active for at least six months since spontaneous remission may occur. If remission is induced by one of these agents, it is necessary to continue maintenance suppressive therapy in order to ensure that the improvement continues.
All drugs in this group are potentially toxic, some extremely so, and therapy should not be embarked upon unless it is evident that all other treatment is ineffective.

Gold - The gold drugs were the earliest of this group to be introduced, and will be discussed in detail in the next section.

D-Penicillamine - Penicillamine is often the first choice drug in this group because it has a greater effect upon the progression of the disease as shown by X-ray changes. It also has a high incidence of side-effects and about 40% of patients have to cease treatment either because of lack of effect or because of toxicity. The incidence of toxic reactions is increased if patients have been previously treated with gold. (74)

Azathioprine - Azathioprine is an anti-metabolite, interfering with purine metabolism, and is thought to have an immunosuppressive effect. It can cause depression of bone marrow activity and have gastro-intestinal intolerance.

Other powerful drugs may be considered but have a high incidence of serious side-effects and are not therefore used unless the disease is very advanced, likely to be fatal and has failed to respond to other treatment. (41)

Steroids
Steroids used systemically in doses necessary to control the inflammation, will inevitably cause Cushing's syndrome and at the same time have no effect upon the eventual outcome. Steroid therapy has no real place in the treatment of rheumatoid arthritis therefore, although prednisolone is occasionally used to provide relief of night pain and morning stiffness. Small doses of prednisolone can also be used in the explosive rheumatoid arthritis which suddenly develops in the elderly. If a response is not observed within a few days of commencing, the treatment should be terminated to avoid steroid dependency.

Local injections of steroids to achieve pain relief in a stiff or deformed joint, or to suppress inflammation in a tendon or ligament, will eliminate the undesirable systemic effects. Subsequent injections are less effective, and a course of more than one or two doses is of no benefit.
1.5 CHRYSOTHERAPY FOR RHEUMATOID ARTHRITIS

1.5.1 Introduction

As was earlier suggested, it was the introduction of gold salts which provided the first sign that rheumatoid arthritis was susceptible to control by drugs. Although the rationale for this step was based upon false assumptions and an associated toxicity has always been recognised, chrysotherapy continues to occupy a significant place in the suppression of the disease.

Notwithstanding a history of more than fifty years' use of gold salts, and recent intensive investigation, our appreciation of their fate in vivo and our understanding of how they exert their anti-rheumatoid action is still incomplete. This section will describe what is known to occur during chrysotherapy using sodium aurothiomalate and also show some of the outstanding problems in order to provide an introduction to the work reported in subsequent chapters.

With the exception of the recently introduced gold phosphine complexes which are orally administered, gold salts are given by intramuscular injection. In adults the dose is not adjusted to the body weight of the patient nor, with few exceptions, has the blood gold concentration been measured to allow modulation of the regime.

During the period prior to the Empire Rheumatism Council (ERC) multi-centre trial, a common system of chrysotherapy evolved among clinicians using sodium aurothiomalate. This system, based upon empirical experience, was adopted for the ERC trial and with certain modifications remains in use today. (36,37).

The concept adopted for the standard regime is that of supplying a total dose of 1.0 g sodium aurothiomalate. A test dose of 10 mg is first given in order to eliminate patients who are particularly sensitive to gold and readily experience toxicity. Subsequent doses are of 50 mg given at weekly intervals until the patient has received the total of 1.0 g.

In the assessment of patients included in the ERC trial which was performed 18 months after the start of the trial, those treated with gold fared better than the controls. (36) At this point some of the gold-treated group, whose improvement was less remarkable, received a second course of injections. In
the final assessment at thirty months, it was noted that much of the initial improvement had disappeared and that the second course of injections (given to the poor responders) did not affect this reversal. (37)

The trial unambiguously showed that gold was effective in controlling rheumatoid arthritis but that a proportion of patients show poor or no response and that eventually active disease reasserts itself. As a consequence therefore it is recognised that maintenance doses of gold must be supplied after the 1.0 g has been given and that a certain flexibility in administration can be introduced. A regime typical of current practice is to give the 10 mg test dose followed by weekly injections of 50 mg. (41) When a favourable response is obtained, the interval between injections is gradually altered to two and then four or six weeks and is continued for an indefinite period. Should a relapse occur while maintenance therapy is being applied, the inter-dose interval is reduced until control is re-established. Non-responders are identified after 1.0 g has been given. If a larger dose, e.g. 100 mg per week for a few weeks still has no effect, chrysotherapy should be discontinued and another drug used.

Blood counts and urinary protein determinations (to identify bone-marrow suppression and kidney damage) should be regularly carried out. Rashes are the most frequent side-effect. Resolution is usually rapid upon temporary cessation of treatment but if the rash reappears when treatment is restarted, it is unwise to continue with chrysotherapy.

1.5.2 Physiology of Gold

Early work using radioactively labelled compounds showed that the absorption of soluble gold complexes by the intestine is very rapid but that plasma concentrations are not comparable with the high levels found following intramuscular injection. This was confirmed by much later work with guinea pigs where the serum gold concentration following oral administration was one-third of that attained by intramuscular injection twenty-four hours after the dose. (75) Urinary excretion is rapid and the gold therefore fails to accumulate and is presumed to be therapeutically ineffective. Therefore, with the exception of the orally administered gold phosphine complexes, gold drugs have been given by intramuscular injection.

Absorption from the injection site into the blood has been frequently measured in patients with rheumatoid arthritis, in healthy volunteers and
in animals, using radioactive and stable gold. Following injection of 50 mg aurothiomalate the peak plasma gold concentrations are reached within six hours. (75-81) The peak is seen somewhat earlier if a smaller injection is given. The increment in plasma gold concentration is extremely variable between individuals, thus although the mean increase is consistently reported as approximately 20 μmol/l (400 μg/100 ml), the range may be as great as 10-30 μmol/l (200-600 μg/100 ml). Some of this variation can be attributed to the different body sizes of the patients. Palmer and Dunkley demonstrated a close relationship between serum gold concentration and body weight in fifteen patients. (82)

The concentration of gold in blood declines from the peak level following an exponential course with a rapid element for thirty hours and a slower fall during the subsequent five days. (77-79,83,84) The mean half life for gold in serum has been calculated and the results are in reasonable agreement at 4.5 to 5.5 days. (77,83,84) A consequence of this rate of decline is that when weekly injections are being given the underlying blood concentration will gradually rise. This trend is illustrated in Fig. 1.3 which shows the rapid increase in concentration immediately after an injection with the fall prior to the next dose. (80,81,85) The underlying concentration eventually stabilises at a level which varies with the individual patient within a range of approximately 5.0 to 17.8 μmol/l (100 to 350 μg/100 ml).

Studies using both 197Au- and 195Au-labelled aurothiomalate showed that a pre-existing gold burden does not influence the way in which injected gold is handled by the body. (77)

The concept of a therapeutic blood concentration is widely applied to pharmacology and was thought to be relevant to chrysotherapy by Lorber. (86) Because of the extreme individual variation in response (i.e. blood gold levels), he suggested that some patients were not getting sufficient gold to the tissues where the disease was active and therefore showed a restrained response to treatment. Likewise, others accumulated an excess of gold and these were the patients in whom gold proved to be toxic. This concept, if demonstrated to be appropriate, provides a place for routine determination of blood gold concentrations and modulation of the dose regime in order to maintain the 'therapeutic level'.

Although no scientific evidence was available to support or refute the idea, many other rheumatologists, on the basis of their clinical experience,
Fig. 1.3 Blood and urinary gold levels from a patient commencing chrysotherapy with sodium aurothiomalate. Amount given by intramuscular injection shown at top. (From Mascarenhas et al. Arth. Rheum. 15, 391-402, 1972).
were unconvinced of the validity of the concept in chrysotherapy. In the next chapter data will be presented from trials which were designed to provide firm evidence as to the relevance of the concept. At about the same time, other workers were carrying out similar exercises.

Distribution of Gold in Blood

During the last decade techniques which make possible a sophisticated separation of blood into component fractions have been developed and applied to the question of how gold is distributed within the blood. At the same time, analysis for gold has become simpler and affords greater sensitivity, making it possible to measure the metal in the minor components of blood.

Early experiments to examine gold distribution were carried out using radioactively labelled colloidal gold but the first attempt to look at sodium aurothiomalate was made by Lawrence. (87) He concluded that plasma gold is exclusively protein-bound, that two-thirds is associated with fibrinogen and that 20% of the total blood gold is within the erythrocytes. However, while the degree of protein binding was found to be high he (and others) inferred that since there is a considerable excretion of gold into the urine following injection, an appreciable fraction must exist for a short time at least, in a small molecular weight form.

Most of Lawrence's conclusions have not been substantiated by subsequent work which has shown that the methods used to investigate gold binding will themselves affect the distribution in blood samples. Relocation of gold has been demonstrated to occur in vitro when samples are left to stand or are subject to a prolonged separation procedure. Furthermore, it is now realised that gold may bind to the membranes used in separation techniques such as dialysis and ultrafiltration with the production of erroneous results. Despite reservations with some experimental data, there are many points where consistent results have been obtained from a variety of techniques.

Contrary to the results obtained by Lawrence, it is no longer believed that gold is found within or attached to erythrocytes and neither is it accepted that there is any difference between the serum and plasma concentrations of gold. (88,89) Lawrence further concluded that gold was firmly bound to plasma protein but this finding also is not entirely correct.
Examination of samples by gel filtration, paper and cellulose acetate electrophoresis, acrylamide gel electrophoresis, immunoelectrophoresis and radial immunodiffusion all show that most of the gold binds firmly to albumin. Depending upon the technique, the proportion of the total gold which is bound to albumin is reported as between 60 (exceptionally) and 99% with most finding 90-95%. (76,90,91)

Again depending upon the procedure and the sensitivity of the technique available to measure gold, the non-albumin component has been found associated with gammaglobulins and with low molecular weight (less than 1000) compounds. (92)

As the total gold concentration is increased, either experimentally in vitro or from patients receiving larger doses of aurothiomalate, so the non-albumin bound proportion also increases. (76,90,93) Because of the considerable technical problems involved in analysing large numbers of samples no study has included more than four patients and there are no results which can be used to attempt to relate non-albumin bound gold to clinical response or toxicity. The most extensive work is that of Danpure et al. (76) where plasma samples were separated into albumin, globulins and unbound fractions. Samples were collected from just three patients at intervals after an injection of aurothiomalate for up to one week. The rapid rise and slow decline in total gold concentration described in the previous section was seen and this was paralleled by the concentration of gold bound to albumin. 10-20% of gold was bound to high molecular weight components and this fraction followed the opposite pattern to albumin with the lowest proportion found after administration. The greatest fluctuation was observed in the non-protein bound gold where the concentration rapidly rose 2 to 5 times within 10-30 minutes of the injection.

Lorber et al. (94) attempted to characterise the high molecular weight components using immunological techniques. They found gold associated with IgG, IgA, IgM, immune complexes and complement proteins and that the gold:protein ratio increased linearly with the total serum gold concentration.

Gold in blood therefore is confined exclusively to the plasma. The majority is bound to albumin with about 10% associated with globulins and
a very small fraction found with low molecular weight compounds. Following an intramuscular injection there is an increase in the unbound and albumin-bound gold with a relative decrease in the proportion of globulin gold. The total gold concentration then decreases exponentially, some gold is excreted, some enters body tissues, and some is redistributed (possibly via other compartments) to the high molecular weight proteins.

Distribution of Gold to Tissues

Tissue distribution of gold following exposure to various gold compounds has been determined in animals and, less extensively, in man. Block et al. (95) gave six different gold compounds as a series of intramuscular injections to rats and measured the concentration of gold in kidney, liver, spleen, heart and lungs at intervals for up to 12 weeks. With the exception of colloidal gold sulphate, the pattern of gold distribution and the change with time was qualitatively similar for each of the gold complexes. One day after the last injection the highest concentration was found in the kidney with substantial amounts also in the liver and spleen. Only trace amounts were found in the other organs examined. The gold concentration in each of the tissues decreased steadily throughout the twelve weeks although the initial pattern of distribution was retained. The quantitative results were quite different for the various compounds with most gold being retained from gold sodium thiosulphate and the least found in animals given sodium succinimidaurate. With colloidal gold sulphate, the gold went initially to the liver but was gradually transferred to the kidney.

Similar results were obtained by Bertrand et al. (96) who also used gold sodium thiosulphate but in both rat and rabbit. These authors examined an increased number of tissues including synovium, tendon and bone and found that connective and non-connective tissues contained gold in similar concentrations. In guinea pigs, rats and rabbits, gold from aurothiomalate was distributed with a similar pattern to that described above. (75,92,97) Most gold went to the kidney with the spleen also having large amounts. Organs of the reticuloendothelial system - liver and lymph glands - had significant concentrations of gold as did the adrenal and reproductive organs. Small amounts were found in all other tissues indicating that absorbed gold becomes very widely distributed. Concentrations were very much lower 24 weeks after the administration.

McQueen and Dykes (92) also administered aurothiomalate to rabbits but differentiated the kidneys into cortex and medulla prior to analysis for
gold. The concentration of the metal in the kidney medulla was equivalent to that measured in liver, adrenal and lung and was between 10 and 20% of that found in the cortex.

In two of these studies artificial inflammation was induced in animals and the amount of gold taken up by the affected tissue was consistently much greater than that taken into normal tissue. (96,97) Other organs, kidney, spleen, liver and heart also had increased concentrations of gold (although not as marked) when compared with normal animals.

This series of reports showed that the tissue distribution of gold from different gold complexes is remarkably similar in different species of animal. The opportunity to conduct similar studies of the distribution of gold in human tissues is unlikely to become readily available. Clearly it is unusual for many patients who have received gold to die and have a full post-mortem with removal of tissue suitable for gold analysis.

Gottlieb et al. (98) were able to measure the concentration of gold in the tissues from one patient, a lady who died from an overdose of barbiturate. The patient had an 11-year history of rheumatoid arthritis and had been treated by chrysotherapy for the previous four years. Therapy had been continued uninterrupted, the last dose was given 8 weeks prior to her death and she had received a total of 2.53 g gold. Nineteen tissues (grouped as reticuloendothelial, musculoskeletal and other) were taken for analysis for gold together with post-mortem blood, urine, plasma, pericardial and synovial fluids. Gold was detected in all samples. The highest measured concentration was in the para-aortic lymphnode with the liver and adrenal also having high concentrations of gold (greater than 100 μg/g). Gold in the kidney cortex was at the same level as the liver and was double that measured in the renal medulla. Low concentrations were found in muscle (except for the site of injection), synovium, cartilage and bone. The authors concluded that gold concentrates in those tissues containing an abundance of reticuloendothelial cells.

This exceptionally extensive work and the authors' conclusions have been referred to repeatedly although there are certain features which should indicate caution in accepting all the data.

1. Six of the tissues were analysed in duplicate and the agreement in results for most of the pairs is so poor as to be beyond acceptance e.g. Results of 396 and 211 μg/g were given for the lymphoid tissue.
2. The plasma concentration (8 weeks after the last injection) was reported as 48.2 µmol/l (950 µg/100 ml) which is in excess of the peak levels usually found a few hours post administration.

3. The urine sample similarly had an impossibly high gold concentration recorded.

These inconsistencies must raise doubts either to the accuracy of the analytical technique (neutron activation analysis) or to the validity of taking samples after an unspecified delay, post-mortem.

A smaller range of tissues from seven patients were obtained for similar analysis by Vernon Roberts et al. (99) Unlike Gottlieb et al. they found the highest concentrations not in reticuloendothelial tissues but in the kidney, adrenal, lymphnode and diseased synovial tissue (i.e. with a similar distribution to that found in experimental animals).

Other opportunities to examine a limited number of tissues are presented when patients undergo surgical operations (e.g. for hip-joint replacement) or from biopsy samples. In one patient so examined the concentration of gold in rheumatoid synovium was at least ten times that in adjacent muscle and skin. (96) A more comprehensive study of gold concentrations in human tissue will be presented in the next chapter.

Excretion of Gold

Given the very high proportion of protein-bound gold found in most studies, urinary excretion of the metal should be trivial. That gold can be measured in the urine during chrysotherapy is however a point used in the argument that, despite the experimental results, some is not tightly protein-bound, particularly soon after an injection.

The urinary excretion increases abruptly during the first day post-injection and thereafter falls steadily during subsequent days (80,100) demonstrating a similar pattern to that seen for plasma concentrations (Fig. 1.3). With the standard weekly dose of 50 mg sodium aurothiomalate, between 10% and 20% of the injected gold is excreted in the urine during the next seven days. When maintenance therapy is being given the proportion excreted progressively falls through the second and following weeks. (80) In general, the greater the amount injected, the greater the percentage of the dose that is excreted.
Monitoring urinary excretion has been suggested as a valuable technique to predict response to chrysotherapy. Smith et al. (101) proposed that patients could be divided into three groups, i.e. normo-, hypo- and hyper-excretors of gold. It was suggested that hyperexcretors remove gold from the body too rapidly or before sufficient has concentrated in tissues to be effective. Conversely hypoexcretors retain excessive gold and toxic side-effects are produced.

This proposal has not been supported by data from other investigators and while the relationship is not strong hyperexcretion is more likely to be associated with high plasma gold concentrations (79,80,83) and not with the low levels as predicted by the hypothesis of Smith et al. (101)

Removal of gold via the faeces is even more variable. Mascarenhas et al. (80) performed gold balance studies with three patients receiving conventional chrysotherapy and with patients on maintenance therapy. With a single exception, removal of gold in the urine was always greater than that through the faeces (an observation confirmed by other reports). Nevertheless the gold excreted in the faeces formed a significant proportion of the total eliminated (between 10% and 55%). One preliminary study with two patients attempted to determine the amount of biliary excretion of gold. It was shown that this accounted for less than 50% of faecal gold. (102).

Thus in the seven post-injection days somewhere between 14% and 40% of the gold is excreted in the urine and faeces. Measurements of gold in hair, skin and nails indicate that insignificant amounts are lost into these tissues. (103) Therefore the body retains in excess of 60% of the injected gold and as has been seen above while uptake into the kidney, spleen and liver accounts for most of this, some gold is retained in all body tissues.

From metabolic balance studies and the use of whole body radiation counting, Gerber et al. (84) followed $^{195}$Au sodium aurothiomalate in patients with rheumatoid arthritis. From their data they formulated a linear two-compartment model. They described a "superficial" compartment which includes serum, synovial fluid and possibly other body fluids also and a "deep" compartment. This so-called deep compartment was recognised to be an undoubted over-simplification of the real situation particularly since the distribution of gold in different organs is not homogeneous. However, the rates of movement of gold into and out of serum can be represented in
1.5.3 Effects of Gold

Toxic responses. The major limiting factor in the use of chrysotherapy is the significant incidence of side-effects. The number of patients reporting undesirable responses to gold is given in the major trials (37, 104,105) at 30-35% (Table 1.4). These effects range from relatively trivial, tolerable reactions to marrow aplasia which can be fatal. A wide range of iatrogenic effects have been attributed to chrysotherapy and these are described below. It is however important to note that the incidence of some of the reactions e.g. skin rashes may not be as high as first appears. In the well-designed trials the number of patients in whom skin eruptions and proteinuria developed was almost as high in the control patients given an innocuous placebo as in the gold treated subjects. (32)

Table 1.4

TOXIC EFFECTS OF GOLD

<table>
<thead>
<tr>
<th>Description</th>
<th>Incidence in patients receiving gold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucocutaneous reactions</td>
<td>15%-30%</td>
</tr>
<tr>
<td>Proteinuria and nephrotic syndrome</td>
<td>3%-7%</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>3%</td>
</tr>
<tr>
<td>Marrow Aplasia</td>
<td></td>
</tr>
<tr>
<td>Miscellaneous reactions</td>
<td></td>
</tr>
<tr>
<td>cholestatic hepatitis</td>
<td></td>
</tr>
<tr>
<td>enterocolitis</td>
<td></td>
</tr>
<tr>
<td>pulmonary infiltration</td>
<td></td>
</tr>
<tr>
<td>ocular chrysiasis</td>
<td></td>
</tr>
<tr>
<td>metallic taste</td>
<td></td>
</tr>
</tbody>
</table>

Mucocutaneous reactions. Effects upon the skin and mucous membranes of the mouth are the most frequently reported toxic reaction to gold. Pruritis is the commonest manifestation followed by non-specific dermatitis. Further lesions which have been described include lichen planus, pityriasis rosea and exfoliative dermatitis. (106) These reactions are found to be mild and self-limiting and upon cessation of gold therapy healing occurs either with topical steroids or more usually without any additional treatments. In most cases the skin reaction first develops early on in a course
of chrysotherapy. When a good response to the removal of gold is obtained, careful therapy can be successfully reintroduced without any further problems.

Proteinuria and nephrotic syndrome. Mild, intermittent albuminuria can be expected to occur in young or middle-aged women from miscellaneous causes such as urinary tract infection and the incidence of proteinuria found in controlled trials is almost as great in controls as in gold treated patients. Patients are usually asymptomatic and the proteinuria detected only by regular monitoring of the urine. Renal damage as a consequence of gold therapy is usually a membranous glomerulonephritis and is much more infrequent than the intermittent albuminuria. (107) Progressive renal impairment and renal failure is avoided by withdrawal of chrysotherapy and an excellent prognosis is possible.

Thrombocytopenia and Marrow Aplasia. The thrombocytopenia associated with chrysotherapy is of two types:

1. an isolated haematological phenomenon with an incidence of about 3% and less serious than,
2. a manifestation of generalised marrow aplasia.

The appearance of isolated thrombocytopenia is unpredictable and regular platelet counts will not necessarily predict thrombocytopenia. Life threatening haemorrhages have not occurred but immediate withdrawal of gold and commencement of steroid therapy is required. (108)

Marrow aplasia is potentially the most serious side-effect. (109) The frequency with which it occurs is not clear. From a review of reported cases it has been suggested that in the United Kingdom, 16 deaths occurred during seven years (110) while other studies examined 50 cases from which the mortality was 66%. (106) A drop in indices of marrow activity is an alarm to cease chrysotherapy and investigate the problem.

Bone marrow examination allows differentiation between isolated thrombocytopenia and aplastic anaemia. In the former condition a cellular marrow with active megakaryocytes is found while in aplasia the picture is of a hypocellular condition with suppression of blast cells and maturation arrest. The number of cases has been too few to allow the preparation of adequate treatment. Chelating agents, steroids and marrow transplantation have been
used but the appreciable number of deaths (from haemorrhage and/or infection) shows that these are far from successful.

Miscellaneous Reactions. Less frequently occurring side-effects have been reported to develop in patients receiving chrysotherapy. Improvement upon removal of the gold and reappearance if therapy is re-instated are the signs which indicate that these effects are associated with gold.

Cholestatic Jaundice. Obstructive jaundice has been described in three middle-aged women patients. (Ill) A typical clinical picture with jaundice, malaise, pruritis etc. developed after two or three injections with sodium aurothiomalate. Drug-induced jaundice was considered after laparotomy or liver biopsy revealed no specific causes. Spontaneous recovery occurred in all three cases.

Enterocolitis. This too is an uncommon reaction occurring in middle-aged women who have received low doses of gold. Symptoms include fever, nausea, vomiting, abdominal pain and diarrhoea. The colon becomes inflamed and has superficial erosions but rapidly heals with withdrawal of gold and the use of steroid enemas. (112).

Diffuse Interstitial Lung Disease. Pulmonary injury producing symptoms of cough and dyspnoea, and having radiological signs of bilateral pulmonary infiltration has been described in several reports during the last five years. Lymphocytes and plasma cells are seen in lung biopsies to infiltrate the alveolar septa and interstitial-fibrotic tissue. (113) In eighteen of the twenty patients reviewed by Scott et al. (114) the lung dysfunction improved or resolved completely.

Foetotoxicity. Foetotoxicity and teratogenicity of gold have not been examined although the advisability of continuing with chrysotherapy during pregnancy has been questioned. (115)

1.5.4 Effects of Gold Upon Immune and Inflammatory Responses.

Possible Mechanisms of Action

The immune response and the inflammatory response represent major components in rheumatoid disease activity. It is, therefore, from the effects of gold upon the many facets of these processes that an appreciation of the mode or modes of action of gold drugs can be gathered. The first studies to answer
some of these questions were unfruitful and failed to demonstrate effects of gold upon humoral or cell-mediated immunity. However more recently, elegant experiments have shown that gold has important modulating activity upon immune and inflammatory responses. (116)

A gradual decrease in the circulating concentration of immunoglobulins and rheumatoid factor in patients treated with gold has been successfully demonstrated on a number of occasions. (94,117) The fall in concentration of these anti-bodies does not begin until treatment has progressed for about six months and then plateaus after one to three years. The decrease compared with pretreatment concentrations was shown to average 53%, 37% and 34% for IgM, IgG and IgA respectively by Lorber et al. (94) who also showed a 27% decrease in the number of circulating lymphocytes and a fall in the rheumatoid factor titre (sometimes to zero).

Concurrent with the decline in immunoglobulin concentration there is an increase in serum albumin levels suggesting that a generalised suppression of protein synthesis is not responsible for this effect of gold salts. (94) Therefore a restriction on the number of cells responsible for synthesis of immunoglobulin and/or actual inhibition of protein synthesis within the cells, could be effecting the gradual decreases observed. From a variety of experimental approaches it is evident that mitogen- and antigen-stimulated lymphocyte cell proliferation is inhibited by sodium aurothiomalate in vitro. (118) By acting upon the T lymphocyte proliferation, activity of the immunoglobulin-producing B lymphocytes (which require T cells as helpers) will be suppressed. Other functions of the lymphocytes e.g. cellular cytotoxicity, migration, phagocytosis, chemotaxis and digestion are also inhibited by gold. (116)

Activation of complement via both the classical and alternative pathways has a profound stimulatory effect upon the inflammatory processes. Sodium aurothiomalate inhibits in vitro activation of Cls and Factor D (both of which act as serine esterases) to shut down the classical pathway and to interrupt the amplification loop of the alternative pathway respectively. (119,120)

The phagocytic activity of inflammatory cells from patients with rheumatoid arthritis is enhanced with controls but is suppressed in those treated with sodium aurothiomalate. (121) Suppression develops progressively throughout
the period of treatment and persists beyond cessation of chrysotherapy. The mechanism of suppression is unknown but it is suggested that there may be (i) inhibition of cellular migration from germinal sites and (ii) membrane stabilisation so that release of lytic enzymes into synovial fluid is prevented.

One of the earliest attempts to determine a mechanism of action for gold in rheumatoid arthritis was described by Persellin and Ziff. (122) They measured the activity of lysosomal enzymes from peritoneal macrophages of guinea pigs and noted that inhibition occurred when gold was added at concentrations of 2.5–5.0 mM which is a 200–500 fold excess compared with expected therapeutic concentrations in synovial fluid but might be achieved as gold is actively sequestered into the lysosome. This inhibition is of considerable importance since the activity of these enzymes, released from macrophages in the joint tissue, is believed to be responsible for the destruction of synovium which in turn presents structurally altered protein capable of reinforcing the immune response. (123) Lysosomal enzymes from other animals and from man which have subsequently been shown to be inhibited by gold are listed in Table 1.5. Included also are the enzymes from serum which have been similarly investigated and it is intriguing to note that gold apparently has no inhibitory action on these enzymes. (124)

Reference to proteolysis causing damage to synovial tissue was made above and there is some evidence that gold salts have an effect upon the synovium itself. In addition to the enzymes concerned with tissue destruction those involved with connective tissue metabolism may be inhibited by gold. (125, 126) The activities of fibroblast glucosamine-6-phosphate synthetase, malic dehydrogenase and enzymes of the nucleic acid synthesis pathways are inhibited by gold as are those of amino acids and carbohydrate metabolism taken from blood cells. Proliferation of cultured synovial cells and the synthesis of collagen by the fibroblasts in culture are altered in the presence of sodium aurothiomalate. Dose dependent inhibition of cell growth and type III collagen synthesis was produced by the gold compound but not by sodium thiomalate. Synovial proliferation and pannus growth are damaging features to joints in rheumatoid arthritis as infiltration of cartilage occurs and mobility becomes impaired and painful. Restriction of synovial cell metabolism and invasive proliferation would therefore provide relief from the disease.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Effect of Gold</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycosidase</td>
<td>Lysosomes</td>
<td>Inhibition of activity</td>
<td>1, 2</td>
</tr>
<tr>
<td>Catheptic protease</td>
<td>Lysosomes</td>
<td>&quot;&quot;</td>
<td>3</td>
</tr>
<tr>
<td>Catheptic protease</td>
<td>Lysosomes</td>
<td>Inhibition of release</td>
<td>4</td>
</tr>
<tr>
<td>Elastase</td>
<td>Lysosomes</td>
<td>Inhibition of activity</td>
<td>5</td>
</tr>
<tr>
<td>Cathepsins</td>
<td>Peritoneal macrophages</td>
<td>&quot;&quot;</td>
<td>6</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>Bovine spleen</td>
<td>&quot;&quot;</td>
<td>7</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>Synovium</td>
<td>&quot;&quot;</td>
<td>3, 8</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>g. pig macrophages</td>
<td>&quot;&quot;</td>
<td>2</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>Rat liver lysosomes</td>
<td>&quot;&quot;</td>
<td>3</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>Synovial fluid</td>
<td>&quot;&quot;</td>
<td>1, 3</td>
</tr>
<tr>
<td>α-N-acetylgalactosaminidase</td>
<td>Synovial fluid</td>
<td>&quot;&quot;</td>
<td>1, 9</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>Serum</td>
<td>No effect</td>
<td>10</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>Serum</td>
<td>&quot;&quot;</td>
<td>10</td>
</tr>
<tr>
<td>α-N-acetylgalactosaminidase</td>
<td>Serum</td>
<td>&quot;&quot;</td>
<td>10</td>
</tr>
</tbody>
</table>

References to Table 1.5

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10. J. Pharm. Exp. Ther. 197 142-152 1976
Fig. 1.4 Sites of action of gold (indicated by * ) upon processes involved in rheumatoid arthritis. Most of the effects take place within the synovial fluid but some may occur elsewhere.
Some of the evidence which demonstrates that gold will affect many parameters of immune and inflammatory responses has been listed. Other actions of gold have been earlier referred e.g. inhibition of protein denaturation and there is continued speculation that involvement with additional metabolic processes may occur thus widening even further the sphere of operation of this anti-rheumatic drug.

It is however important to note that most of the actions of gold described have been demonstrated only in \textit{in vitro} systems or in artificially induced models of inflammation and the relevance and relative importance of each of these observations to the \textit{in vivo} disease is very much uncertain. However in these experimental systems the effects (when tested) were shown to be dose-dependent and to be caused by the gold and not by thiomalate. A schematic representation of the possible actions of gold in rheumatoid arthritis is shown in Fig. 1.4 which brings together theories of the aetiology of the disease, the immune mechanisms and the pathological lesions i.e. the gross, chronic, inflammatory response.

1.6 AIMS AND OBJECTIVES

The incidence of gold toxicity together with those patients who fail to show a therapeutic response presents a vexing problem during chrysotherapy. The work reported in subsequent chapters describes investigations carried out to examine features which were considered to be of possible importance in patient response to chrysotherapy. These features included the concentrations of gold in body fluids and tissues, the role of the carrier molecule (thiomalate) upon uptake, disposition and elimination of gold and trace-metal interrelationships and possible mechanisms of gold toxicity.

1. A spectrum of clinical response, related to rates of absorption, metabolism, excretion etc. is not uncommon in pharmacology. Consequently the importance of regular measurements of biologically active compounds, e.g. lithium, or some closely related metabolite, is well recognised and therapeutic drug monitoring is now standard practice. Clinical investigations were therefore planned to determine whether regular measurements of the gold concentrations in body fluids would assist in the management of chrysotherapy for rheumatoid arthritis.
2. Passage of the injected drug from the site of injection to the site of action has not been thoroughly elucidated. Relevance of the thiomalate (or other carrier) moiety and the tissue or plasma concentration of thiol-containing compounds upon reactivity, rate of transfer etc. are unknown. Experiments to determine movement of gold and thiomalate in experimental animals were conducted and the concentration of gold in major organs measured in gold treated animals and in patients receiving chrysotherapy.

3. Essential metals, particularly copper, are important in the aetiology of rheumatoid arthritis (Fig. 1.2) and chrysotherapy provokes changes in serum metal concentrations. As fluctuations in trace metal dispositions are associated with the toxicity of other metals the effect of chrysotherapy upon the metal content of tissues and body fluids of experimental animals was assessed.
CHAPTER TWO

SODIUM AUROTHIOMALATE AND
CLINICAL STUDIES
2.1 INTRODUCTION

2.1.1 Routine Monitoring of Blood and Urine Gold Concentrations During Chrysotherapy

The extended background to the development of chrysotherapy as an acknowledged treatment for tuberculosis, rheumatoid arthritis and later for other conditions was discussed in the previous chapter. Phases such as the introduction of different gold drugs, evolution of therapeutic regimes, conduct of organised trials each extended over many years.

Because of this background of more than fifty years' use, the quantitative and qualitative incidence of therapeutic failure has become assimilated into the tradition of chrysotherapy and is accepted almost as an inevitable feature of treatment by gold. Teaching practices devote as much time to the recognition of, and response to, toxic manifestations as is given to the methods of assessment of effects upon the actual disease. Furthermore, the incidence of toxicity means that chrysotherapy is accepted almost as a treatment of last resort when all else has failed. Patients in whom gold fails to elicit any beneficial response are left with little alternative other than powerful analgesics.

The overall effect is that for these different reasons between one-fifth and one-third of patients whose condition is deemed appropriate for chrysotherapy are eventually denied the considerable therapeutic benefit which may be derived from treatment with gold.

The gross manifestations of toxicity, dermatitis, proteinuria, thrombocytopenia etc. are well recognised and were described in the first chapter. However, what causes some patients to react to gold in a particular way that is not seen in others is not at all understood. Similarly the lack of clinical response to gold cannot be explained when it appears that other patients in whom the disease (as judged by clinical examination, radiological assessment and laboratory investigations) is very similar, demonstrate dramatic improvement in their besetting condition.

This was the problem that obtained for many years in the field of chrysotherapy for rheumatoid arthritis and was addressed first by
Smith et al. (101) then subsequently by a few others but in particular by Lorber. The hypothesis proposed in these early investigations was that the serum total gold concentration was fundamental to the response obtained. Toxicity would be associated with high serum gold concentrations while a lack of response would be found when low serum gold concentrations were achieved. Central to this concept was the assumption that given a standard administration of gold it is the rate of urinary excretion which has the major influence upon the concentration of gold in the serum. It was suggested therefore that the difference between patients which was responsible for the variation in response during chrysotherapy was the renal handling of gold.

To differentiate between the patients who might demonstrate toxicity, might not respond or might benefit from chrysotherapy, Smith et al. proposed that urinary gold excretion in samples collected over twenty-four hours, on seven successive days, should be monitored in order to select the hypo-, hyper- and normo-excretors of gold. (101) However, the collection of twenty-four hour urine samples is notoriously difficult and to anticipate many patients properly doing so for extended periods is extremely optimistic. At the same time, the problems of accurate analysis for low concentrations of gold meant that adoption of the proposals of Smith et al. by any other than a limited number of specialist centres was not practically possible.

A somewhat more sophisticated procedure was advocated twelve years later by Kralik. (127) Because of the improvements in analytical techniques it became possible to measure gold relatively simply in serum and in urine. Kralik proposed that the ratio, concentration of gold in urine/concentration of gold in serum, would provide a valuable indicator for the prediction of complications in chrysotherapy. He suggested that a low ratio (less than 1.0) was indicative of hypo-excretion and an accumulation of gold in the serum. Where patients were found to have a ratio of less than 1.0 he advocated appropriate modification to the course of treatment. At about the same time Lorber first suggested adjustment of treatment in order to maintain the gold concentration above a certain (unspecified) level and thereby attain a therapeutic response. (128)
Of these original reports it was only that of Smith et al. which included results of continual observations on patients receiving adjusted-dose therapy (101) but for the reasons discussed above this type of work was never followed up or repeated elsewhere. With the development of atomic absorption spectrophotometry as a reliable analytical procedure for accurately measuring gold, and with the suggestion by Lorber that measurements of serum gold concentrations could also be of value in the management of rheumatoid arthritis patients treated with chrysotherapy, it was decided to carry out a study to assess the value of undertaking routine determinations of blood and urine gold concentrations.

2.1.2 Maintenance Chrysotherapy in Rheumatoid Arthritis

The conventional regime for the administration of gold salts is to give weekly injections of 50 mg sodium aurothiomalate (25 mg gold), or a similar dose of aurothioglucose, until a total dose of 1.0 g (500 mg gold) has been provided. Thereafter the patient is transferred to maintenance therapy and the interval between injections is increased, usually to four weeks or a little longer. (129) This regime is still widely used by many rheumatologists but there are those for whom the concept of a 1.0 g initial course is a little too inflexible and who prefer at times to adopt either a gentler or a more aggressive approach depending upon the response by the patient. (41)

Similarly the management of the maintenance period has been questioned and reconsidered with conflicting results obtained from a wide range of different schedules. Although there is a well-recognised time-lag between treatment and response - both at commencement and termination of chrysotherapy, the results reported by Cats would suggest that maintenance therapy is unnecessary. (130) From the results of this study it was inferred that remission is maintained for at least a year after the loading dose of 1.0 g has been received. While this conclusion is consistent with the findings of the Empire Rheumatism Council (ERC) trial (36), the author apparently overlooks the observations in the final ERC report where virtually all the advantages accrued from gold treatment had been lost at the end of two years. (37) While a number of studies have been conducted where a more active course of therapy has been applied (41, 81, 85, 131, 132) few have actually been designed or have presented their results in such a way as to allow comparison with the standard, four-weekly regime.
Of those where such a comparison is possible completely contradictory results were obtained. Thus Mascarenhas et al. grouped patients into those with substantial therapeutic response, those who improved but also developed toxic manifestations and those without therapeutic response. (80) Within each group there were a similar number of subjects on weekly, bi-weekly and 4-weekly maintenance therapy and an appropriate range of plasma gold concentrations.

On the other hand, Lorber et al. who specifically modulated maintenance therapy in order to keep serum gold concentrations in excess of 15.2 \( \mu \text{mol/L} \) (300 \( \mu \text{g/100 ml} \)) claimed not to have an increased incidence of adverse reactions coupled with significant clinical benefits recorded in those patients compared with the control group receiving the conventional dosage schedule. (85)

Because these studies produced such disparate results and recommendations it was considered appropriate to critically compare maintenance dose schedules. A study was therefore undertaken to determine the response to gold in patients given injections at two-weekly intervals and at four-weekly intervals with particular attention given to progression of the disease, the incidence of toxicity and the relationship of serum gold concentrations to relapse and side-effects.

2.1.3 Tissue Gold Levels After Chrysotherapy

From the work in animals given soluble gold salts, it is apparent that following injection the gold is rapidly dispersed throughout all the body. (92,95-97) Certain tissues however accumulate gold to greater concentrations than others and this particularly applies to the kidney cortex, the liver, spleen and possibly the adrenal gland. Disposition of gold has been followed at various time intervals up to twenty-four weeks after injection but in most cases animals have been examined after just a few days.

Progressive loss of gold from all tissues occurs with time and at the completion of the prolonged (24 week) study only about 1% remained compared with the amount present at two weeks. (97) The exception to this was the gold content in the lymph glands which was 20% of the earlier level.
The data available from human tissues is far less extensive, only the report from Gottlieb et al. attempted to provide a picture of the total body distribution. (98) This however referred to just one patient and contained certain analytical anomalies which were discussed in the preceding chapter. In this report the lowest concentrations of gold were found in the tissues of the skeleto-muscular system - bone, synovium, muscle and cartilage.

From patients undergoing surgery it is possible to obtain and take for analysis, samples of tissue which are exposed or excised during the operative procedure. While this will not necessarily provide data for a wide range of tissues, the opportunity is presented to assemble relevant data from a reasonable number of patients.

It was felt important to know something more of the gold content of articular tissue in order to help appraise the role of gold salts in the treatment of rheumatoid arthritis. The animal studies are not informative on this aspect and the data of Gottlieb et al. (98) remain somewhat unreliable. Therefore in a series of rheumatoid arthritis patients for whom surgical hip joint replacement was provided as part of the management of their condition, samples of bone, synovium, striated muscle and fat were analysed for the gold concentrations.

2.1.4 Additional Studies

Opportunities arose during the course of this work to examine briefly two other problems associated with chrysotherapy:

1. Possible foetotoxicity of gold therapy during pregnancy.

Possible Foetotoxicity of Gold

Teratogenic effects have been demonstrated experimentally following exposure to very large concentrations of gold. (133) Little data are available concerning the effects in man of gold administered therapeutically during pregnancy. Two cases relevant to this question required the measurement of gold in body tissues and fluids thus adding to the available information.
Cases of marrow aplasia manifesting as a thrombocytopenia are a rare complication of chrysotherapy but while aplasia is uncommon, mortality occurs with a probable incidence of at least 50% in such cases. (106) In a statistical study of drug associated deaths compared with the number of prescriptions written, fatal gold-induced thrombocytopenia was cited as having the greatest frequency of occurrence. (110)

To reverse the thrombocytopenia, the use of chelating agents or peritoneal dialysis have been advocated in order to remove the offending gold. However, the elimination of gold may (134) or may not (135) be effective. Similarly a place for marrow transplantation has been considered but has not been successfully demonstrated. (136) The severity and danger of thrombocytopenia therefore makes not only careful supervision mandatory for all patients treated with gold but also requires continued investigation of methods which might improve understanding of this toxic response and allow more effective treatment.

Two cases of ladies who developed refractory thrombocytopenia after receiving short courses of gold provided for the monitoring of serum and urine gold concentrations, and other parameters throughout the period of chelation therapy.

2.2 CLINICAL METHODS

2.2.1 Introduction

The clinical studies were carried out in association with the Department of Rheumatology, Guy's Hospital, Norfolk and Norwich Hospital, St. Richard's Hospital, Chichester, The Poisons Unit, New Cross Hospital and the United States Air Force Hospital, Lakenheath, (USAFE), Suffolk. The staff at these centres contributed considerably to these studies in the organisation and management of the clinical programmes, evaluation of patients' progress and collation of data from various sources.

2.2.2 Routine Monitoring of Blood and Urine Gold Concentrations During Chrysotherapy

The study was designed to determine which, if any of a variety of clinical and laboratory parameters, including gold levels, were associated
with a favourable response to chrysotherapy. Patients included in the study were grouped into two separate categories:

1. Retrospective Group.

All patients (total number = 86) who attended the Guy's Hospital Department of Rheumatology Gold Clinic during a period of one and a half years were included in this study. Forty patients were also included in a separate study which examined the incidence of skin reactions and their association with serum gold concentrations.

2. Prospective Group.

New patients to the Clinic (total number = 22), who had never previously been treated with gold, were followed throughout the initial one gram course with sodium aurothiomalate.

As each patient entered the studies the following information was obtained: age, sex, duration of disease and the presence or absence of: nodules, erosions on X-ray examination and rheumatoid factor in serum.

At each visit to the clinic the patient was examined by the physician and samples of blood and urine (random sample of mid-stream urine) were collected. Gold injections were given at the clinic after blood and urine samples had been taken.

Treatment of Samples:-

Two samples of blood were collected - anticoagulated (using dipotassium ethylene-diametetra-acetic acid) and clotted blood. The clotted blood samples were centrifuged and the serum transferred to a clean tube.

Analysis:-

Whole blood. The following haematological parameters were measured red cell count, white cell count, haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haematocrit, platelet count and erythrocyte sedimentation rate.

Serum. The gold concentration was measured.

Urine. Qualitative analysis for protein, glucose, ketones, bilirubin, blood and pH. Quantitative determination of gold and creatinine.
At the end of the one-gram course, patients in the Prospective group were graded according to their clinical response as - much worse, worse, unchanged, improved, much improved.

2.2.3 Maintenance Chrysotherapy in Rheumatoid Arthritis

This study was designed to compare the response to maintenance gold injections given at two-week intervals and at four-week intervals by monitoring progress of the disease and incidence of toxicity and to examine the relationship between response and serum gold concentrations.

Stage 1. Patients receiving their first course of chrysotherapy were admitted to the preliminary phase of the study which consisted of a test dose of 10 mg sodium aurothiomalate followed by weekly injections of 50 mg to a total of one gram. Non-steroidal, anti-inflammatory agents were continued throughout the study and alterations, other than reduction of dose on remission, were avoided when possible.

At the commencement of the study, patients were randomly allocated to proceed during stage 2 to either the 2-week or 4-week maintenance schedule. Each group was matched for age, sex and X-ray score.

Stage 2. Those patients who had shown definite clinical improvement by the third month of stage 1, and had developed no evidence of gold toxicity, progressed to the second stage of the study which then continued for a further twelve months.

Patients were examined after 3, 4 and 5 months of weekly gold injections (Stage 1.) and at three-monthly intervals during maintenance therapy i.e. at 8, 11, 14 and 17 months from the commencement of the study. Observations made at each examination were:--

duration of morning stiffness, joint tenderness, grip strength and proximal interphalanged joint circumference. X-ray examination for erosions in the hands and feet were made at 5, 11 and 17 months.

Blood samples were collected at each assessment point. Haemoglobin and ESR were measured on whole blood while serum samples were analysed for sheep cell agglutination titre and gold concentration.
Sixteen patients with rheumatoid arthritis who had previously received gold injections were included in this study. The subjects were undergoing surgical replacement of the hip-joint to relieve pain and improve mobility. During the course of the operation tissue samples were removed into clean plastic containers. The full list of tissues taken from the patients was: bone (acetabulum), skeletal muscle, synovium, fat, skin, joint capsule, bone marrow and tendon. Specimens of all these tissues were not obtained from every patient.

2.2.5 Additional Studies

Possible Foetotoxicity of Gold

Case 1. A 28 year old woman received four injections of gold, for the treatment of psoriatic arthropathy, during the first part of her second pregnancy. Spontaneous onset of labour occurred at 36 weeks and a baby boy (weighing 2160 g) was delivered by elective Caesarian Section because the mother had had a previous section. There were no other complications and the infant's condition at birth was good without requiring resuscitation.

Case 2. A woman in whom rheumatoid arthritis had been diagnosed when she was aged 17 years, commenced gold therapy two years later and was receiving 100 mg sodium aurothiomalate monthly. At the age of 21 she became pregnant but against the advice of her clinician decided to continue with the chrysotherapy throughout the pregnancy. The last injection of gold given while she was pregnant was at three days prior to delivery.

Laboratory Investigations in Gold-Induced Thrombocytopenia

Case 1. A lady (EA) aged 60 years had rheumatoid arthritis diagnosed three years previously and was treated with analgesics. Chrysotherapy was considered suitable and was commenced in April 1979. After three months, by which time she had received 670 mg sodium aurothiomalate she suddenly developed a widespread petechial rash, a burning feeling in hands and feet, haematuria and her urine contained traces of protein. Gold treatment was stopped and she was admitted to hospital where gold toxicity was considered. Haematological examinations of the blood and bone marrow confirmed a thrombocytopenia and absence of marrow mega-karyocytes.
Case 2. A 49 year old lady (J.G.) had an eighteen month history of rheumatoid arthritis. She was originally treated with physiotherapy and Ibuprofen but at the assessment one year after diagnosis, radiological deterioration of the disease was apparent and it was decided to institute chrysotherapy. An improvement in her condition was noted but after two months she complained of blisters on her hands. This was thought not to be associated with gold but a reduced dose was prescribed. One month later however she required admission to hospital for treatment of a generalised rash, pruritis and mouth ulcers, diagnosed as probably gold induced. A thrombocytopenia was discovered when investigated on admission.

2.3 ANALYTICAL METHODS

2.3.1 Gold

The concentrations of gold in serum, urine and tissue samples were determined by atomic absorption spectrophotometry. Two atomic absorption spectrophotometers were used for the measurement of gold. The instrumental conditions used are shown in Table 2.1. Hollow cathode lamps obtained from S. & J. Juniper, Harlow, were used in both instruments. A stoichiometric, air/acetylene flame was appropriate for the gold determinations.

<table>
<thead>
<tr>
<th>Table 2.1 Instrumental Operating Conditions Used For The Analysis of Gold By Atomic Absorption Spectroscopy</th>
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<tbody>
<tr>
<td>Perkin Elmer Instrumentation Laboratories</td>
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<tr>
<td>Model 303 Model 353</td>
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<tr>
<td>Lamp current (mA)</td>
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<tr>
<td>Wavelength (nm)</td>
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<tr>
<td>Slit width</td>
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<tr>
<td>Photomultiplier Voltage (V)</td>
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<tr>
<td>Scale expansion - serum</td>
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<tr>
<td>- urine</td>
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<tr>
<td>- tissues</td>
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</table>
A modification of the method described by Lorber et al. was used. (128) The technique of internal standardisation was adopted to calculate the concentration of gold and a detergent solution was added to the serum in order to reduce viscosity and facilitate sample aspiration through the nebuliser and premix chamber. Methodological details are:

Reagents: Stock gold chloride standard, 1mg Au/ml. The atomic absorption standard solution from BDH Chemicals Ltd. was obtained.
Working gold standard 5µg/ml. 0.5 ml of the stock gold solution was diluted to 100 ml with distilled water.
Triton X-100, 5% v/v. 25 ml Triton X-100 (Sigma Chemicals Limited) was added to 200 ml distilled water. The mixture was warmed to effect solution and the volume made up to 500 ml with distilled water.

Two tubes (A and B) were set up for each specimen.

<table>
<thead>
<tr>
<th></th>
<th>Tube A</th>
<th>Tube B</th>
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<tbody>
<tr>
<td>Serum</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Working gold standard</td>
<td>-</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>5% Triton X-100</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

The contents of each tube were thoroughly mixed, the solutions aspirated in turn into the atomic absorption spectrophotometer and the absorbance noted.

Calculation of concentration of gold, µmol/l = \( \frac{250 \times A \times 10}{(B - A) \times 197} \) where

A = absorbance for Tube A
B = absorbance for Tube B

Urine

The internal standardisation procedure used for serum was modified to determine gold in urine. Addition of the detergent solution to reduce viscosity was not necessary but because of the lower concentration of gold within the urine, a greater sample to diluent ratio was provided.
Two tubes (A and B) were set up for each specimen.

<table>
<thead>
<tr>
<th></th>
<th>Tube A</th>
<th>Tube B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Working gold standard</td>
<td>-</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.5 ml</td>
<td>-</td>
</tr>
</tbody>
</table>

The contents of each tube were thoroughly mixed, the solutions aspirated in turn into the atomic absorption spectrophotometer and the absorbance noted.

Calculation of concentration of gold, \( \mu \text{mol/l} \) = \[
\frac{4 \times 125 \times A \times 10}{3 \times (B - A) \times 197}
\]

where

\( A \) = absorbance for Tube A

\( B \) = absorbance for Tube B

To ensure that reliable, accurate results were obtained, an internal quality control (QC) programme was devised for this laboratory and an external quality assessment (QA) scheme was established with the participation of six other laboratories.

Internal quality control: 500 ml donor horse serum was obtained from SeraLab Limited. 200 ml were dispensed into two clean 250 ml volumetric flasks and an appropriate volume of diluted gold standard solution added to give concentrations of 7.6 \( \mu \text{mol/l} \) (150 \( \mu \text{g}/100 \text{ ml} \)) and 17.8 \( \mu \text{mol/l} \) (350 \( \mu \text{g}/100 \text{ ml} \)). The volumes were made up to 250 ml, dispensed into clean, glass 5 ml tubes and stored at -20°C.

Whenever clinical specimens were analysed, one each of the two internal QC samples were allowed to thaw, were mixed and also analysed for the concentrations of gold. Results were reported only if the values obtained on the internal QC samples fell within specified ranges (± 7.5% at 7.6 \( \mu \text{mol/l} \) and ± 5.0% at 17.8 \( \mu \text{mol/l} \)).

External quality assessment: Samples were similarly prepared from donor horse serum but with a wider range of gold concentrations. Each month, three samples were sent to the participating laboratories and their results returned to the organising centre (University of Surrey). From this data the mean, standard deviation and coefficient of variation were calculated. Although the number of participants was small making the calculated mean somewhat suspect this programme aids rapid detection and
quality assessment scheme for gold formed part of a larger programme which included the analysis of copper, zinc and aluminium in serum, cadmium and lead in blood and cadmium and mercury in urine.

Tissue Samples

Two sample preparation procedures were used during the period that this work was in progress. The samples obtained from the study reported in section 2.4.3 were subjected to ashing in a muffle furnace while the later samples, section 2.4.4, were treated with strong oxidising acids.

Dry ashing: Tissue samples weighing about 0.5 g were added into tared silica crucibles and accurately weighed. To obtain representative specimens, small tissue snips from several sites within the material removed at operation were taken to give the total weight of about 0.5 g. Segments of bone were obtained by sectioning the acetabulum in the longitudinal plane to expose the spongy interior tissue. Small sections were removed from across the face of the cut surface and from the outer edges, thus providing samples from several areas of the bone. Duplicate samples were taken from all specimens.

The crucibles containing the tissue samples were placed inside a cold muffle furnace and then heated to 450-500°C for at least 16 hours. The heat was turned off and the furnace allowed to cool to enable removal of the crucibles. A white powdery ash remained although with bone some of the original architecture of the inorganic matrix could still be seen. These residues, including that of the bone, were readily dissolved in 1.0 ml of 0.1 M nitric acid.

Standard gold solutions: The stock standard solution was diluted 1.0 ml to 100 ml with 0.1 M nitric acid to provide a gold solution of 10 µg/ml. A further series of dilutions were made to give standard gold solutions of 1.0, 2.5, 5.0 and 7.5 µg/ml.

The standard and sample solutions were sequentially aspirated into the atomic absorption spectrophotometer and the absorbances noted.

Standard curves were constructed from the readings and the concentrations of gold in the sample solutions determined. The amount of gold in the original sample of tissue was calculated from the formula \[ x = \frac{C}{w} \]
where \( x \) = tissue gold concentration in \( \mu g/g \) wet weight
\( c = \) concentration of gold in sample solution in \( \mu g/ml \)
\( w = \) weight of tissue sample taken for analysis in grams.

Acid digestion. Tissue samples weighing 0.5 to 1.0 gram were transferred to tared, acid-washed, 100 ml conical flasks and accurately weighed. As for dry ashing, small pieces of tissue were taken from various sites to obtain representative specimens. Duplicate samples were weighed out for all specimens.

Concentrated nitric acid (5.0 ml) and 60\% perchloric acid (1.0 ml) were added to the samples using automatic dispensers (all acids were Analar Grade). The flasks were maintained at room temperature for at least 30 minutes to allow any initial reaction and frothing to subside and were then transferred to a hot plate placed within a wash-down fume cupboard. The surface temperature of the hot plate was set to 100\( ^\circ \)C and the flasks gently heated for about one hour. After all the tissue samples had dissolved the temperature was gradually increased to about 150\( ^\circ \)C. Excessive heating was avoided to prevent the nitric acid from boiling away before oxidation of the organic material was complete. If any of the solutions darkened, the flasks were removed and left to cool, a further 5.0 ml concentrated nitric acid added, and then returned to the hot plate. When a pale yellow or colourless solution was obtained the temperature was increased to heat the sample to dryness. The flasks were then removed from the hot plate and left to cool. Two reagent blank flasks were prepared with each batch of samples and carried through the entire digestion and analytical procedures.

The inorganic residues were redissolved by the addition of 1.0 ml aqua regia (conc. nitric acid: conc. hydrochloric acid; 1:1) and very gentle warming. Distilled water was then added to give a final volume of 5.0 ml.

Standard gold solutions. Gold solutions of 1.0, to 7.5 \( \mu g/ml \) were prepared similarly to that previously described but to contain 20\% v/v aqua regia.

The standard and sample solutions were aspirated into the atomic absorption spectrophotometer and the absorbances noted. Additional washing with water through the nebuliser was performed between samples to avoid possible damage due to the high acid concentration of these solutions. Potential
difficulties associated with the viscosity of concentrated acids were not apparent and sample flow through the nebuliser remained satisfactory.

The concentrations of gold in the sample solutions were determined by reference to a calibration curve prepared from the readings obtained from the standard solutions and the gold content of the samples of tissue calculated from the formula.

\[ x = \frac{c \times 5}{w} \]

where

- \( x \) = tissue gold concentration in μg/g wet weight
- \( c \) = concentration of gold in the sample in μg/ml
- \( w \) = weight of the tissue sample taken for analysis in grams.

2.3.2 Urine Tests

Urine screening tests were performed during attendances at the Rheumatology clinics and the commercially available test strips (Ames) were used to monitor pH, protein, glucose, ketones, bilirubin and blood.

2.3.3 Haematology

The routine haematological investigations - haemoglobin, red cell count, white cell count, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haematocrit were determined by the standard laboratory method using a Coulter Counter. The platelet counts were similarly measured in the counter and the erythrocyte sedimentation rate was determined according to the Westergren procedure. (137)

2.3.4 Rheumatoid Factor

The titre of rheumatoid factor was measured at Guy's Hospital by the sheep cell agglutination test in serum samples at dilutions of \( 1/16 \) to \( 1/1024 \). Agglutination was assessed visually.

2.3.5 Clinical Parameters

Quantitative clinical measurements incorporated in these studies included an articular index for the assessment of joint tenderness and a score of the erosions present in bone.

The index of joint tenderness used was that devised by Ritchie et al. (138) The response by the patient to firm pressure exerted over the articular margin or to movement of the joint, was observed. Where there was no
tenderness a score of 0 was recorded. A complaint of pain scored +1, pain plus wincing scored +2 and pain, wincing and withdrawing counted +3. The cumulative scores from all joints examined gave the overall articular index. Ritchie et al. demonstrated that the interindividual variation in results was quite wide but that one observer could reliably obtain consistent results. (138) Therefore all clinical assessments in any one study were carried out by the same person.

To monitor the changes in disease activity affecting the bones the erosions observed on radiological examination of the hands and feet were counted. (139)

2.4 RESULTS AND DISCUSSION

2.4.1 Routine Monitoring of Blood and Urine Gold Concentrations During Chrysotherapy

Retrospective Study

Eighty-six patients with rheumatoid arthritis were included in the retrospective study. In 74 of these subjects, samples of both serum and urine were collected during the same visit to the clinic, on at least 5 separate occasions (Table 2.2). The correlation between serum and urine gold concentrations from these temporally matched samples was statistically significant in 28 (38%) of the cases. When additional data were available, a stronger correlation was evident. Thus for 39 of the patients, ten or more pairs of data were available and significant correlations were found in 26 (67%) of the sets of results. This striking degree of correlation found in a number of patients is illustrated in Fig. 2.1.

Table 2.2 Correlation Between Serum and Urine Gold Concentrations in Retrospective Study

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<table>
<thead>
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<tbody>
<tr>
<td>Total number of patients</td>
<td>86</td>
</tr>
<tr>
<td>Number with at least 5 pairs of serum and urine results</td>
<td>74</td>
</tr>
<tr>
<td>Number with significant correlation (p&lt;0.05) between pairs of results</td>
<td>28 (38%)</td>
</tr>
<tr>
<td>Number with at least 10 pairs of serum and urine results</td>
<td>39</td>
</tr>
<tr>
<td>Number with significant correlation (p&lt;0.05) between pairs of results</td>
<td>26 (67%)</td>
</tr>
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</table>
Fig. 2.1  Serum and urinary gold concentrations measured at regular intervals in a patient receiving sodium aurothiomalate at the doses shown and demonstrating the correlation between serum and urine gold levels.

Gold injections (mg)

Serum gold (μmol/l)

Urine gold (μmol/mmol creatinine)
Prospective Study

In this part of the study 22 patients were followed throughout the initial one gram course of chrysotherapy. At the completion of the course, the clinicians' and the patients' assessment of response was used to subdivide the group. No patients suffered a set-back or deterioration in condition. Seven had shown no response and were unchanged, seven were considered to have improved and eight were thought to have benefited markedly.

The mean gold concentrations, for serum and urine, were calculated from all the results obtained from all the patients within a particular response group. There were no significant differences in either the serum or the urine gold concentrations between any of the groups (Table 2.3).

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<thead>
<tr>
<th>Response Group</th>
<th>Number of Patients</th>
<th>Serum (μmol/l)</th>
<th>Urine (μmol/mmol Creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unchanged</td>
<td>7</td>
<td>6.70 ± 3.20</td>
<td>2.49 ± 1.07</td>
</tr>
<tr>
<td>Improved</td>
<td>7</td>
<td>10.46 ± 2.59</td>
<td>3.03 ± 0.96</td>
</tr>
<tr>
<td>Much Improved</td>
<td>8</td>
<td>7.21 ± 3.30</td>
<td>2.79 ± 1.03</td>
</tr>
</tbody>
</table>

Values are reported as mean ± standard deviation.

Data is from prospective group in the investigation of routine monitoring of blood and urine gold concentrations during chrysotherapy. The patients had never previously been treated with gold and were followed throughout the initial one gram course with sodium aurothiomalate. Samples were collected at the weekly visit to the Rheumatology Clinic before the injection of gold.

The correlation between pairs of serum and urine results was examined as in the retrospective study. In those patients who responded to gold and whose condition was improved the correlation was greater than in those in whom chrysotherapy was ineffective (Fig. 2.2). This difference however was not statistically significant.
Fig. 2.2 Significance of correlations between serum and urine gold levels (as shown in Fig. 2.1) versus clinical response to chrysotherapy in patients included in prospective study.

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of patients</th>
<th>% with significant correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unchanged</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Improved</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Much improved</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>43</th>
<th>43</th>
<th>75</th>
</tr>
</thead>
<tbody>
<tr>
<td>% with significant correlation between serum and urine gold levels</td>
<td>a = not significant</td>
<td>b = p&lt;0.05</td>
<td>c = p&lt;0.01</td>
</tr>
</tbody>
</table>
Examination of the clinical parameters recorded on the patients according to their therapeutic response, is shown in Table 2.4. There was no difference in sex distribution, duration of the disease, pretreatment levels of ESR or rheumatoid factor and the pretreatment presence of erosions, between the three clinical response groups. There was a trend, which was not statistically significant, towards a less favourable response with increasing age. However, the presence of nodules was significantly correlated with failure to respond to chrysotherapy.

In those patients who showed excellent response to gold, the signs of improvement were apparent earlier (i.e. after a lower total dose) than was found in patients whose response was less marked. Improvement was noted in the first group after a mean dose of 252 ± SD 145 mg compared with 493 ± SD 184 mg in the second group. This difference was statistically significant (p<0.025).

Of the 40 patients examined for the incidence of dermatological side-effects, 14 (35%) developed rashes (eczematous-8; maculo-papular-3; lichenoid-2; pityriasiform-1) accompanied by either local or generalised pruritis. The mean serum gold concentration was not significantly different from that of the full group. The rashes lasted from three weeks to one year. No cases of bone-marrow depression developed in any of these patients.

The rationale for the routine monitoring of serum and/or urine gold concentrations during chrysotherapy would be that the results may help to improve the efficacy of treatment and diminish the incidence of side-effects. This argument presupposes that:

(a) a favourable response requires a certain gold concentration in the serum,

(b) toxicity is a consequence of increased gold concentration in the serum, and

(c) an increased urinary excretion of gold causes a reduction in the serum gold concentration (see Fig. 2.3a).
Table 2.4 Clinical Parameters in Patients in the Retrospective Study

<table>
<thead>
<tr>
<th>Condition</th>
<th>Number of patients</th>
<th>Sex M/F</th>
<th>Age years</th>
<th>Disease duration years</th>
<th>ESR mm/h</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unchanged</td>
<td>7</td>
<td>3/4</td>
<td>53 ± 6</td>
<td>8.0 ± 6.8</td>
<td>46 ± 20</td>
<td>1:128</td>
</tr>
<tr>
<td>Improved</td>
<td>7</td>
<td>2/5</td>
<td>46 ± 16</td>
<td>9.3 ± 8.2</td>
<td>34 ± 14</td>
<td>1:32</td>
</tr>
<tr>
<td>Much Improved</td>
<td>8</td>
<td>4/4</td>
<td>42 ± 13</td>
<td>7.1 ± 7.5</td>
<td>59 ± 34</td>
<td>1:64</td>
</tr>
</tbody>
</table>

ESR (erythrocyte sedimentation rate)
SCAT (sheep cell agglutination titre)
Values are reported as mean ± standard deviation
a p<0.05 compared with unchanged group
Fig. 2.3 Proposed relationships between urinary excretion of gold and serum gold concentration.

(a) Serum concentration inversely proportional to urine excretion. Smith et al. (101).

(b) Urine excretion proportional to serum concentration.
This is the hypothesis which was first elaborated by Smith et al. (101) and later reintroduced by Kralik (127) and Lorber et al. (128). The number of patients actively followed throughout a period of chrysotherapy to verify these suppositions were very few. Isolated reports of cases which were inconsistent with the hypothesis also appeared however. Thus Jessop described two patients who developed blood dyscrasias but whose mean serum gold concentrations (at one week post-injection) were no higher than those measured in the other eleven members of the group. (140) Jones et al. measured urinary excretion in 48 patients receiving chrysotherapy and found only one who had a urinary gold excretion which was considerably in excess of the others and none who had particularly low rates of excretion. (100) These results do not correlate with the incidence of failure to respond or of toxicity reported in large trials.

On the other hand two reports (78,79) suggested that the hypothesis may not be entirely inappropriate. Krusius et al. found that patients who eventually developed signs of toxicity had higher levels in the serum and urine while those with a poor response to sodium aurothiomalate had lower gold concentrations in both fluids. (79) Serum and urine gold levels were positively correlated i.e. were consistent with Fig. 2.3b but not with 2.3a. The samples were collected during the seven days following the injection which brought the total dose to 150 mg. They did not attempt to monitor levels on a regular basis. Jessop and Johns found no correlation between mean serum and urine gold concentrations in patients who were being regularly monitored. However, they did find that subjects who developed serious skin reactions (such that chrysotherapy had to be stopped) had significantly higher serum gold concentrations. (78) At the same time there were no correlations between serum gold and other toxic side-effects.

The data reported here were obtained from the first study where a large series of patients were routinely monitored throughout prolonged (up to twelve months) periods of chrysotherapy with measurements of serum and urine gold concentrations in addition to the usual parameters of disease activity. The results obtained are not in accord with any of the tenets of the Smith-Kralik-Lorber hypothesis. Clinical improvement was seen to occur irrespective of the pre-injection levels of gold in serum. There were patients who did not benefit from chrysotherapy but whose serum gold concentrations were in excess of levels in patients who improved markedly.
(Table 2.3). Furthermore there was no correlation between side-effects and the concentration of serum gold. The only iatrogenic effects found in these patients were in a small group who developed skin rashes. These subjects were not exceptional in having high gold levels. The long-term pattern of serum or urine concentrations, e.g. sudden or periodic fluctuations in level, also failed to reveal any distinction between patients responding in different ways to exposure to gold.

The positive correlation found between serum and urine concentrations of gold suggests that it is unlikely that urinary excretion influences the concentration of gold in serum (as represented in Fig. 2.3a) but that in a stable situation something approaching a constant proportion is transferred from the serum to the urine (Fig. 2.3b).

That the correlation between gold levels in serum and urine is stronger in the patients included in the retrospective study (most of whom were at the stage of maintenance therapy) is consistent with the results obtained during the prospective study. As is shown in Fig. 2.2 it is the patients who responded favourably to gold, and who would be expected to transfer on to maintenance therapy, who had the best correlation while patients in whom chrysotherapy was unsuccessful and would possibly cease gold treatment showed poor correlation.

The results reported here are consistent with the early reports of Jones et al. (100) and Jessop (140) and are also in agreement with most of the subsequent data which has examined the relationship between serum gold concentrations and therapeutic response. Only in somewhat unusual circumstances has there been any suggestion by these workers that a correlation between the body burden of gold, (but not the serum levels) and the response, might exist. With very high doses of sodium aurothiomalate (two to four times the conventional amounts), Rothermich et al. (131) were able to obtain improved clinical status in patients previously resistant to gold while Furst et al. (132) found a fivefold increase in the incidence of severe side-effects among a high-dose group of patients.

The work of Lorber et al. (85,86,142,143), which followed from the earlier speculations of this author (128), remains isolated therefore from the main stream of evidence referred to previously. He reports that
patients with higher serum gold concentrations are more likely to respond with favourable developments of their rheumatoid disease. These patients were said to notice benefit earlier and for a more prolonged period and to experience greater changes in the parameters of disease activity. At the same time they suggested that there was no increased incidence of adverse reactions compared with the patients having lower serum gold levels although it should be noted that the total incidence, at 40-50% of patients, is higher than that found in most other trials.

The study reported here, also failed to reveal any other laboratory indices which could be used as alternatives to the serum or urine gold concentrations to predict patient response to chrysotherapy. The most useful guides to a successful outcome were found to be the absence of nodules and earlier signs of gold having some effect. The physician therefore can rely upon very little external help when deciding which patients should be treated with gold, whether treatment could be improved by modifications to the course of therapy and if toxic side-effects will suddenly become evident.

Of the features of the Smith-Kralik-Lorber hypothesis, the proposal that it is urinary excretion which modulates serum levels of gold, cannot be accepted when the data included in Table 2.2 and in numerous other reports are presented. The concept of a therapeutic range for total serum gold concentrations during chrysotherapy is similarly opposed by the evidence shown in Table 2.3 and by all other workers with the exception of Lorber.

2.4.2 Maintenance Chrysotherapy in Rheumatoid Arthritis

At the start of the study to compare maintenance doses there were 58 patients included but 14 were forced to withdraw before or soon after entering the second phase of the investigation (Table 2.5). The remaining 44 were divided into the 2-week group (21 patients) and the 4-week group (23 patients). All completed at least six months of maintenance chrysotherapy but six were withdrawn before the end of the study because of toxic complications. Symptoms of less severe toxicity were experienced by a further 11 patients who were nonetheless able to continue with the treatment. The frequency of different toxic reactions is shown in Table 2.6. Relapse in previously improved clinical conditions occurred in five patients between eight and eleven months of chrysotherapy.
Table 2.5 Clinical Outcome of Patients Involved in Maintenance Chrysotherapy Study

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Dose frequency during maintenance therapy (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>No. admitted to study</td>
<td>58</td>
<td>-</td>
</tr>
<tr>
<td>No. proceeding to maintenance therapy</td>
<td>44</td>
<td>21</td>
</tr>
<tr>
<td>No. completing 6 months of maintenance therapy</td>
<td>44</td>
<td>21</td>
</tr>
<tr>
<td>No. completing 1 year of maintenance therapy</td>
<td>38</td>
<td>17</td>
</tr>
<tr>
<td>No. developing toxicity</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>No. of relapses</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2.6 Distribution and Frequency of Side-Effects During Maintenance Chrysotherapy Study

<table>
<thead>
<tr>
<th>Toxic Reaction</th>
<th>Dose frequency during maintenance therapy (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Rash</td>
<td>6</td>
</tr>
<tr>
<td>Mouth Ulcer</td>
<td>3</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
</tr>
<tr>
<td>Withdrawals</td>
<td>4</td>
</tr>
</tbody>
</table>

All patients had shown some improvement in general condition during the preliminary phase of weekly injections. The essential clinical details at this stage are summarised in Table 2.7 which shows that both treatment groups were matched. Clinical (morning stiffness, articular index, grip strength and proximal interphalanged joint circumference), laboratory (haemoglobin, ESR and gold) and X-ray measurements were also similar with no significant differences between the two groups (Tables 2.8 and 2.9).
Table 2.7 Clinical Details of Patients Followed During One Year of Maintenance Chrysotherapy - Details at start of study

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of patients</th>
<th>Sex M/F</th>
<th>Age years</th>
<th>Disease Duration years</th>
<th>Rh. Factor</th>
<th>Nodules Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-week injections</td>
<td>21</td>
<td>9/12</td>
<td>54 ± 10</td>
<td>5.8 ± 5.0</td>
<td>11(52%)</td>
<td>7(33%)</td>
</tr>
<tr>
<td>4-week injections</td>
<td>23</td>
<td>7/16</td>
<td>55 ± 12</td>
<td>7.0 ± 5.7</td>
<td>12(52%)</td>
<td>7(30%)</td>
</tr>
</tbody>
</table>

Values are reported as mean ± standard deviation.

Table 2.8 Clinical Measurements in Patients Followed During One Year of Maintenance Chrysotherapy - Details during maintenance period

<table>
<thead>
<tr>
<th>Dosing frequency (weeks)</th>
<th>Maintenance Injection (Months)</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morning stiffness (mins)</td>
<td>2</td>
<td>25 ± 55</td>
<td>41 ± 67</td>
<td>46 ± 78</td>
<td>35 ± 52</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>12 ± 18</td>
<td>17 ± 30</td>
<td>11 ± 20</td>
<td>20 ± 37</td>
</tr>
<tr>
<td>Articular index (score)</td>
<td>2</td>
<td>6.5 ± 7.3</td>
<td>6.6 ± 7.8</td>
<td>5.7 ± 7.7</td>
<td>6.1 ± 8.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.7 ± 3.5</td>
<td>3.5 ± 3.9</td>
<td>3.2 ± 3.5</td>
<td>3.2 ± 2.8</td>
</tr>
<tr>
<td>Grip strength sum of 2 hands (mm Hg)</td>
<td>2</td>
<td>325 ± 136</td>
<td>322 ± 139</td>
<td>324 ± 141</td>
<td>329 ± 151</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>292 ± 138</td>
<td>299 ± 135</td>
<td>307 ± 141</td>
<td>314 ± 140</td>
</tr>
<tr>
<td>PIP joint circum. (mm)</td>
<td>2</td>
<td>595 ± 52</td>
<td>597 ± 53</td>
<td>593 ± 53</td>
<td>592 ± 55</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>571 ± 42</td>
<td>566 ± 42</td>
<td>577 ± 43</td>
<td>572 ± 45</td>
</tr>
</tbody>
</table>

PIP (proximal interphalangeal)

Values are reported as mean ± standard deviation.

Patients received 50 mg sodium aurothiomalate at two or four-weekly intervals throughout the period of maintenance chrysotherapy.
Table 2.9 Laboratory Measurements in Patients Followed During One Year of Maintenance Chrysotherapy - Details at start of study

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of patients</th>
<th>Haemoglobin g/100 ml</th>
<th>ESR mm/hr</th>
<th>Serum gold µmol/l</th>
<th>X-ray score</th>
<th>Morning Stiffness</th>
<th>Articular index</th>
<th>Grip strength</th>
<th>PIP joint circumference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2—week injections</td>
<td>21</td>
<td>13.3 ± 1.7</td>
<td>27 ± 21</td>
<td>14.2 ± 4.9</td>
<td>20 ± 37</td>
<td>25 ± 38</td>
<td>7.0 ± 7.7</td>
<td>317 ± 142</td>
<td>597 ± 53</td>
</tr>
<tr>
<td>4—week injections</td>
<td>23</td>
<td>12.4 ± 1.2</td>
<td>27 ± 22</td>
<td>17.8 ± 6.0</td>
<td>19 ± 20</td>
<td>12 ± 18</td>
<td>4.7 ± 3.7</td>
<td>291 ± 137</td>
<td>583 ± 49</td>
</tr>
</tbody>
</table>

Values are reported as mean ± standard deviation
The clinical improvements noted during the period of weekly injections were generally sustained during maintenance chrysotherapy in both groups. Some parameters however did tend to regress a little as the inter-injection interval was lengthened. The duration of morning stiffness showed an increase towards the middle of the maintenance period which was exaggerated in the two-week group but then improved again over the latter stage of the study (Fig. 2.4). The articular index of joint tenderness improved slowly but steadily in both groups (Fig. 2.4) as did the grip strength (Fig. 2.5). It might be expected that the effects of the disease upon the hands and fingers would influence both grip strength and joint swelling at the same time. This however, would appear not to be so, for while grip strength increased in both groups and the 2-week group had a slightly better response than the 4-week, the proximal interphalanged joint circumferences remained virtually unchanged throughout and were of similar size in both groups (Fig. 2.5). Therefore, grip strength and joint swelling must be seen as two distinct indices of the disease. These long-term trends are displayed by the presentation of mean values in Figs. 2.4 and 2.5. The full data are tabulated in Table 2.9 which reveals the large standard deviations indicative of considerable spread of results. Differences between the two groups therefore failed to reach significance as did any changes during the course of treatment compared with the pre-maintenance therapy results.

After twelve months maintenance therapy one patient from each group had reduced number of nodules while four from the two-weekly and two from the four-weekly groups had increased numbers.

Laboratory and X-ray Measurements

There was no change in haemoglobin concentrations throughout the active period of chrysotherapy studied (Fig. 2.6). By contrast, erythrocyte sedimentation rates altered considerably during the investigation. There was a steady decline during the preliminary phase which reflected the general improvement in the patients condition. The decline in ESR was continued through the first six months of maintenance therapy in those patients receiving gold at two-weekly intervals but then gradually increased again and at the end of the study the mean values were similar for both treatment groups (Fig. 2.6). The serum gold concentration fell
Fig. 2.4 Changes in joint tenderness and morning stiffness throughout the investigation of maintenance chrysotherapy. Points represent mean values and summarise data in Tables 2.8 and 2.9. Interinjection intervals during maintenance chrysotherapy were 2 weeks (21 subjects) or 4 weeks (23 subjects).
Fig. 2.5 Changes in grip strength and proximal interphalangeal (PIP) joint circumference throughout investigation of maintenance chrysotherapy. Points represent mean values and summarise data in Tables 2.8 and 2.9. Interinjection intervals during maintenance chrysotherapy were 2 weeks (21 subjects) or 4 weeks (23 subjects).
Fig. 2.6 Changes in ESR and blood haemoglobin concentrations throughout the investigation of maintenance chrysotherapy. Points represent mean values and summarise data in Tables 2.9 and 2.10. Inter-injection intervals during maintenance chrysotherapy were 2 weeks (21 subjects) or 4 weeks (23 subjects).
as anticipated following cessation of weekly injections but levels were stable soon after three months maintenance therapy. The patients receiving two-weekly injections had values 25% greater than the patients in the other group (Fig. 2.7).

Rheumatoid factor titre (as reported from Guy's Hospital), fell in patients from both treatment groups. Three of those in the more frequent dose and two receiving the four-weekly injections eventually had undetectable levels of rheumatoid factor. A further five of the patients with two-weekly injections had lower titres without becoming seronegative. The change in rheumatoid factor titre was significantly greater (p<0.005) in the two-week compared with the four-week group.

Very gradual radiological deterioration continued in patients from both groups with an equal distribution (Fig. 2.7). The X-ray score increased in 23 patients although in nineteen cases this was with five or fewer additional erosions and only one patient (on the two-week course) had an increase of greater than ten erosions. Radiological improvement with the loss of one or more erosions occurred in five patients.

As with the clinical measurements the full data have been tabulated and are shown in Table 2.10.

The patients whose condition suddenly deteriorated towards the end of the period studied were not characterised by any obvious clinical or laboratory features. The serum gold concentrations were the same in these patients as in their cohorts and there were no signs predictive of impending therapeutic failure (Table 2.11).

The purpose of this investigation was to determine whether maintenance therapy administered at more frequent intervals would confer benefit to the patients by improving the rheumatoid condition without producing additional toxicity. More frequent injections will necessarily produce an increased body load of gold and higher mean serum gold concentrations which have been argued by Lorber to be crucial to successful chryso-therapy.
Changes in serum gold concentrations and X-ray scores throughout the investigation of maintenance chrysotherapy. Points represent mean values and summarise data in Tables 2.9 and 2.10. Injection intervals during maintenance chrysotherapy were 2 weeks (21 subjects) or 4 weeks (23 subjects).

**X-ray score**
- ○-----○ 2-weekly dose group
- ○-----○ 4-weekly dose group

**Serum gold (μmol/l)**
- ■-----■ 2-weekly dose group
- □-----□ 4-weekly dose group

**Fig. 2.7**
Table 2.10 Laboratory and Radiological Measurements in Patients Followed During One Year of Maintenance Chrysotherapy—Details during maintenance period

<table>
<thead>
<tr>
<th>Dosing frequency (weeks)</th>
<th>Maintenance Injections (Months)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Haemoglobin (g/100 ml)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>13.5 ± 1.6</td>
</tr>
<tr>
<td>4</td>
<td>12.6 ± 1.1</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>23.0 ± 18.0</td>
</tr>
<tr>
<td>4</td>
<td>27.0 ± 19.0</td>
</tr>
<tr>
<td>Serum gold (µmol/l)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.7 ± 4.9</td>
</tr>
<tr>
<td>4</td>
<td>6.0 ± 1.9</td>
</tr>
<tr>
<td>X-ray score</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are reported as mean ± standard deviation

Patients received 50 mg sodium aurothiomalate at two or four-weekly intervals throughout the period of maintenance chrysotherapy.

Table 2.11 Clinical Details of Patients Who Relapsed During Maintenance Chrysotherapy. Serum Gold Levels at 6 Months Compared With Non-Relapsing Cohorts

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of relapsing patients</th>
<th>Sex (M/F)</th>
<th>Mean age (years) (range)</th>
<th>Mean serum gold (µmol/l) (range)</th>
<th>Mean serum gold of non-relapsing patients (µmol/l) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-weekly dose</td>
<td>3</td>
<td>3/0 (47-68)</td>
<td>59</td>
<td>8.12 (5.28-12.39)</td>
<td>5.48 (2.54-14.87)</td>
</tr>
<tr>
<td>4-weekly dose</td>
<td>2</td>
<td>1/1 (34-58)</td>
<td>46</td>
<td>4.62 (3.50-5.74)</td>
<td>5.99 (3.30-9.90)</td>
</tr>
</tbody>
</table>
The results obtained indicated that a maintenance dose of 100 mg sodium aurothiomalate per month, administered in divided doses at two-weekly intervals confers no advantage over 50 mg per month given as a single injection. The signs of benefit demonstrated by clinical and laboratory measurements during the preliminary phase of weekly injections up to a total dose of one gram were generally sustained by patients from both treatment groups and there was a subtle trend towards further improvement as evidenced by results of grip strength and articular index.

The initial response in ESR suggested at first that the frequent administration of gold might induce a more favourable eventual outcome but this was not confirmed by the later results. Similarly the different serum gold concentrations between the two groups was not related to any clinical, laboratory or radiological measurement of response. This is consistent with the results from the study reported in the previous section and fails to substantiate the approach advocated by Lorber that improved response is associated with the maintenance of higher serum gold concentrations.

Patients receiving the two-weekly injections of gold experienced more episodes of toxicity than did those in the lower dose group although there was no significant relationship with serum gold concentration. Furst et al. also noted a higher incidence of toxicity in patients treated with 150 mg of sodium aurothiomalate compared with those given 50 mg. (132) These patients however also received injections with greater frequency which may also be a factor relevant to the development of undesirable side-effects. These authors also were unable to demonstrate a relationship between the incidence of side-effects and serum gold concentrations. (132)

Incidence of relapse during chrysotherapy is not a feature remarked upon in many other studies. Relapse in the five patients in this investigation was not related to frequency of maintenance injections nor to the serum gold concentration. No clinical features singled them out from other patients and all had done well prior to the set-back. That some patients will relapse even after prolonged periods of complete remission and despite continued chrysotherapy has been previously observed (144) with one report that deterioration eventually overtakes most patients and that only 15% will continue chrysotherapy beyond four years. (145)
The results reported here have shown that the management of maintenance therapy is not improved by increasing the rate of administration of sodium aurothiomalate from once to twice per month. Furthermore, the incidence of toxic symptoms increases with the two-weekly injections compared with the conventional procedures.

It is evident that further studies are necessary to determine the long-term response to chrysotherapy in patients receiving gold for several years. If relapses are to be avoided consideration may have to be given to changes in the maintenance therapy regimes as the periods of treatment extend beyond one year. This problem does not yet appear to have been addressed.

2.4.3 Tissue Gold Levels After Chrysotherapy

The gold concentrations in the samples of tissue removed from patients undergoing elective surgery are summarised in Table 2.12. The considerable deposition of metal in synovial tissue is immediately apparent and the amounts found were more than five times those present in other sites. The spread of results obtained was very wide as is evidenced by the standard deviations calculated from the data. Therefore, for further examination of the results, the patients were divided into two groups according to whether or not they were receiving chrysotherapy at the time of operation.

<table>
<thead>
<tr>
<th>Tissue Sample</th>
<th>Synovium</th>
<th>Bone</th>
<th>Muscle</th>
<th>Fat</th>
<th>Skin</th>
<th>Joint Capsule</th>
<th>Bone Marrow</th>
<th>Tendon</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>11</td>
<td>10</td>
<td>11</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Mean (µg/g)</td>
<td>11.07</td>
<td>1.48</td>
<td>1.89</td>
<td>1.24</td>
<td>1.89</td>
<td>0.91</td>
<td>1.48</td>
<td>0.95</td>
</tr>
<tr>
<td>S.D. (µg/g)</td>
<td>20.76</td>
<td>1.62</td>
<td>1.87</td>
<td>1.44</td>
<td>3.60</td>
<td>1.02</td>
<td>0.17</td>
<td>1.34</td>
</tr>
</tbody>
</table>

Results are reported as mean ± standard deviation
Group I included the patients currently receiving gold and Group II consisted of patients who had not received gold for some years. Fortuitously the mean total doses of gold administered to the groups were similar. The two groups were compared by determining the ratio between gold in the synovium and in either muscle, bone or fat. Not all ratios between all tissue pairs were available for all patients.

Table 2.13 Comparison of gold levels in synovium and striated muscle removed at operation. Chrysotherapy was provided recently (Group I) or several years previously (Group II).

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean interval since last-injection</th>
<th>No. of samples</th>
<th>Mean total gold given (g)</th>
<th>Mean tissue gold (μg/g) synovium</th>
<th>Mean tissue gold (μg/g) muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4.2 weeks</td>
<td>5</td>
<td>1.46</td>
<td>23.82</td>
<td>2.96</td>
</tr>
<tr>
<td>II</td>
<td>4.1 years</td>
<td>6</td>
<td>1.38</td>
<td>0.45</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Table 2.13 shows the gold results in the paired samples of synovium and striated muscle. The gold ratio between these tissues in Group I was 8.05:1 whereas in the group no longer receiving gold, the ratio was 0.45:1. This difference in ratio was highly significant, p<0.01 using Wilcoxon's nonparametric ranking test. The gold levels in the pairings of synovium with bone and synovium with fat samples are shown in Tables 2.14 and 2.15 respectively. The ratios of mean gold concentrations in all three sets of tissues are listed in Table 2.16.

Table 2.14 Comparison of gold levels in synovium and bone removed at operation. Chrysotherapy was provided recently (Group I) or several years previously (Group II).

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean interval since last injection</th>
<th>No. of samples</th>
<th>Mean total gold given (g)</th>
<th>Mean tissue gold (μg/g) synovium</th>
<th>Mean tissue gold (μg/g) bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3.6 weeks</td>
<td>5</td>
<td>2.13</td>
<td>19.26</td>
<td>2.20</td>
</tr>
<tr>
<td>II</td>
<td>10.3 years</td>
<td>4</td>
<td>2.19</td>
<td>0.80</td>
<td>0.87</td>
</tr>
</tbody>
</table>
Table 2.15 Comparison of gold levels in synovium and fat removed at operation. Chrysotherapy was provided recently (Group I) or several years previously (Group II).

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean interval since last injection</th>
<th>No. of samples</th>
<th>Mean total gold given (g)</th>
<th>Mean tissue gold (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5.4 weeks</td>
<td>4</td>
<td>1.97</td>
<td>20.14</td>
</tr>
<tr>
<td>II</td>
<td>3.17 years</td>
<td>4</td>
<td>1.19</td>
<td>1.76</td>
</tr>
</tbody>
</table>

Table 2.16 Ratios between Mean Tissue Gold Levels for Synovium with Muscle, Bone and Fat

<table>
<thead>
<tr>
<th>Group</th>
<th>Ratio of synovial gold to:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Muscle</td>
</tr>
<tr>
<td>I</td>
<td>8.05:1</td>
</tr>
<tr>
<td>II</td>
<td>0.45:1</td>
</tr>
</tbody>
</table>

| p     | <0.01 | N.S. | N.S. |

Data taken from Tables 2.13 - 2.15

Differences between Groups I and II tested using Wilcoxon's non-parametric ranking list.

In addition to the patients included in this established study, tissues which were removed at post mortem autopsy from two other gold treated subjects, were also examined.

Details concerning the duration of chrysotherapy and data of last dose were not available for one (R.C.) and the second (E.A.) had been extensively treated with chelating agents following the appearance of gold-induced thrombocytopenia (see Section 2.4.4). However, the results are included (Table 2.17) in order to allow comparison with those shown in Table 2.12 and with those obtained from the literature.
Table 2.17 Concentration of Gold in Tissues (µg/g) Removed at Post-mortem from Two Patients

<table>
<thead>
<tr>
<th>Subject</th>
<th>Synovium</th>
<th>Bone rib</th>
<th>Bone spine</th>
<th>Muscle</th>
<th>Fat</th>
<th>Skin</th>
<th>Renal medulla</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC</td>
<td>0.37</td>
<td>-</td>
<td>-</td>
<td>0.80</td>
<td>0.27</td>
<td>0.30</td>
<td>0.37</td>
<td>-</td>
</tr>
<tr>
<td>EA</td>
<td>1.49</td>
<td>4.71</td>
<td>4.23</td>
<td>2.82</td>
<td>0.84</td>
<td>3.52</td>
<td>-</td>
<td>3.48</td>
</tr>
</tbody>
</table>

The tissue concentrations of gold found (Table 2.12 to 2.17) show very little correlation with those previously reported by Gottlieb et al. (98). The subject (M.V.) described by these authors had received 2.53 g of gold with the last injection eight weeks previously and would therefore be comparable with the patients in Group I in Tables 2.13 to 2.16. Synovium from the knee of M.V. had a reported gold concentration of 32 µg/g which is consistent with the concentrations reported here but for the bone (11 µg/g) and muscle (7 µg/g) the results of Gottlieb et al. (98) are somewhat higher than those shown in Tables 2.13 and 2.14 while for other tissues e.g. skin, bone marrow, renal medulla and liver, their results are 30 to 40 gold greater than are reported here.

The results of Vernon-Roberts et al. are more consistent with those presented here. (99) These authors measured gold in samples obtained at autopsy. With one exception who was still receiving gold, all had ceased chrysotherapy at least two years previously and as long as 23 years before removal of samples. While the tissues examined do not exactly correspond to those included here, the pattern of concentration is similar with very high levels in the synovium and much lesser amounts in bone marrow, skin etc. The highest levels however were found in the adrenal and gonads which were not examined in any of the patients in this report.

The difficulty with the results of Gottlieb et al. (98) was referred to in the previous chapter and a subsequent paper by the same authors further adds to the confusion. In determining the excretion of gold into the hair, nails and skin they were able to detect the metal in all three, even in control subjects who had never received gold therapy. Other investigators (146) have been unable to measure gold in skin samples from control subjects and the amounts measured in patients are consistent
with those shown in Tables 2.12 and 2.17. (146) Therefore in the absence of supporting data from any other investigators, the analytical results of Gottlieb et al. (98) appear to be suspect.

Factors which will influence the concentration of gold found in body tissues include the total amount of metal injected and the latent period between the last injection and analysis. Since both groups in this study received the same amount of gold this factor does not apply when comparing results and one can therefore examine the effect of time upon the disposition of gold. While division of the data into a greater number of subgroups, each representing different times for the latent period, would have been preferable to obtain a clearer picture of long term changes such groups would have had only a small number of samples and not necessarily yielded reliable information. Therefore just the two groups were chosen.

During active chrysotherapy the fraction of injected gold which is retained in the body is selectively concentrated in synovial tissue (the data of Vernon-Roberts et al. (99) indicates that adrenal and gonadal glands also have this capacity) although all other tissues also retain some of the metal. These observations accord with the findings in laboratory animals following injection of gold where selective deposition is also seen to be superimposed upon a widespread, generalised distribution.

Continuing excretion of gold following cessation of injections indicates the slow removal of gold from the initial sites of storage with the consequent lower tissue concentrations measured in specimens obtained from the patients of Group II. At the same time however the reduced ratio of synovial to non-articular tissue gold levels in this group (Table 2.16) suggests that removal of gold from synovial tissue is greater than from other types of tissue.

From these results it can be proposed that in patients with rheumatoid arthritis a labile gold pool exists (which includes the synovium) into which gold enters and is discharged more rapidly than occurs with a widespread pool characterised by a very slow turnover of gold. The long half-life of this second pool is such that gold may still be detected in tissues of some patients more than twenty years after treatment has ceased.
Results from experiments to determine gold disposition in animals were consistent with the existence of a labile gold pool. In rats, considerable concentration of gold occurred at the sites of artificial inflammation (granuloma pouch) but during succeeding weeks the gold rapidly disappeared. (97) While availability of tissue limits the determination of synovial gold in rats there are also sites such as kidney and spleen where initially very high concentrations are quickly depleted.

Specific histochemical examination of synovial tissue also lends support to this hypothesis. Microscopic demonstration of gold in synovial tissues removed at surgery reveals large deposits in the lining cells during active chrysotherapy but that in patients no longer receiving gold, a progressive diminution of concentration occurs with time and the concurrent appearance in deeper connective tissue of gold-rich macrophages. This movement of gold however was limited to tissue taken from diseased joints for synovium that was free from rheumatoid disease contained very little visible gold. With this technique gold deposits were also observed at other sites where macrophage populations were large e.g. kidney, spleen, adrenal. (99)

It therefore appears that during chrysotherapy gold is deposited in the cells of the synovium - probably taken from the gold present in synovial fluid and in plasma and also transported by macrophages. After chrysotherapy has been stopped synovial gold concentration decreases and the visible gold deposits are lost. The reason for the sudden movement of gold is not clear but migration of cells into deeper tissues or removal by macrophages may be responsible for the translocation.

2.4.4 Additional Studies

Possible Foetotoxicity of Gold

Case 1. Exposure to gold occurred when the mother received chrysotherapy, for psoriatic arthritis, early in the pregnancy. For the first 8 hours after the birth the baby had a respiration rate of 70-80 per minute but there was no evidence of recession or distress. He was then noticed to be slightly jittery and gradually deteriorated over the next nine hours becoming pale and distressed with gasping respiration and unrecordable blood pressure. The results of investigations carried out at the time were:-
Haematocrit 40.6%, white cell count 18,300 per mm$, platelets $160 \times 10^9$ per litre, sodium 138 mmol/l, potassium 7.2 mmol/l, chloride 101 mmol/l, urea 12.5 mmol/l, blood glucose 1.4 mmol/l, Chest X-ray - patchy changes, heart normal size; abdominal X-ray - no abnormalities. Blood culture - no growth.

A provisional diagnosis of septicaemic shock was made and treatment with antibiotics and intravenous fluids was commenced. Intubation and mechanical ventilation was also established.

A blood clotting screen (Table 2.18) revealed the presence of a coagulation problem and it was decided to perform an exchange transfusion with fresh blood and fresh frozen plasma. The baby sustained cardiac arrest just as the exchange transfusion was completed. This was corrected by massage and bicarbonate. A lumbar puncture at this time gave a clear yellow fluid with no pus.

<table>
<thead>
<tr>
<th>Results</th>
<th>Reference or Control Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>0.75 g/l</td>
</tr>
<tr>
<td>Prothrombin time</td>
<td>29 secs</td>
</tr>
<tr>
<td>Partial thromboplastin-kaolin time</td>
<td>145 secs</td>
</tr>
<tr>
<td>Thrombin time</td>
<td>17 secs</td>
</tr>
<tr>
<td>Fibrin degradation products not detected</td>
<td></td>
</tr>
</tbody>
</table>

The condition of the baby then improved and a systolic blood pressure of 54 mm Hg was recorded. A second clotting screen also showed improvement and a repeat of the chest X-ray showed diffuse patchy shadows in both lungs. However, a few hours later he developed pulmonary haemorrhages which continued over the next four hours until he died at 57 hours of age.

A post mortem examination revealed extensive pulmonary bleeding and an intracranial haemorrhage. Samples of liver, kidney and lung tissue were taken for gold determination and the results are shown in Table 2.19.
The concentration in the kidney (0.33 µg/g) is of the same order as was found in patient R.C. (Table 2.17) and a little lower than those reported by Vernon-Roberts et al. (99) two or three years after ending gold treatment. Gold in the liver (0.37 µg/g) is about one tenth the amount measured both in EA (Table 2.17) and by Vernon-Roberts et al. The results obtained by Gottlieb et al. (98) were very much higher for both these tissues. There are little data against which to compare the gold concentration in the lung (0.46 µg/g). Only Vernon-Roberts et al. have reported results which were 0.5, 3.0 and 5.0 µg/g from patients last receiving gold 20, 3 and 0 years ago respectively. (99)

Table 2.19 Gold Measurements Associated with Two Cases of Exposure to Gold in utero

<table>
<thead>
<tr>
<th>Case</th>
<th>Samples</th>
<th>Gold concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Liver</td>
<td>0.37 µg/g</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>0.33 µg/g</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>0.46 µg/g</td>
</tr>
</tbody>
</table>
| 2    | Maternal serum at delivery: 19.9 µmol/λ
|      | Cord serum at delivery: 11.4 µmol/λ
|      | Infant's serum at two-and-a-half weeks: 2.6 µmol/λ|

Case 2. Exposure to gold in this case occurred throughout the pregnancy which proceeded uneventfully to term when the mother delivered a baby girl weighing 2970 g. There were no obvious major or minor abnormalities evident on the infant. A venous blood sample was collected from the mother at delivery together with a simultaneous umbilical cord blood sample. When the baby was two-and-a-half weeks old a venous blood sample was taken from her. The serum gold concentrations are also shown in Table 2.19.

The cord blood gold concentration, 11.4 µmol/λ (245 µg/100 ml), indicates that levels very similar to those measured during active chrysotherapy are attained in the foetal circulation when the mother is treated during
pregnancy. The decrease in gold over the next two weeks, to 2.6 μmol/l (51 μg/100 ml), demonstrates that the neonate has no difficulty with elimination of gold, the fall in serum concentration being similar to that observed following an injection in patients on maintenance chrysotherapy.

There have been no animal studies designed to assess the effect of gold compounds given at therapeutic levels upon the foetus but at concentrations considerably in excess of those used for chrysotherapy, sodium aurothiomalate has teratogenic effects in rats and rabbits. (133) It is known that gold administered in conventional amounts will cross the human placenta. Some measurements of gold in perfusion fluid from isolated placentae have been made in this laboratory as part of a different project. An albumin-colloidal gold complex was administered and gold transferred to the foetal circulation. Rocker and Henderson, using electron micro-probe analysis, detected gold in the liver and kidney but not in other tissues examined, from a 20 week aborted foetus. (115) Quantitative measurements were not attempted.

The number of documented cases of women receiving or continuing chrysotherapy throughout pregnancy are very few. The two cases reported here therefore are valuable additions to the available information concerning gold and foetotoxicity.

The foetus aborted by Rocker had no obvious abnormality upon external examination and preservation of tissue for histological assessment was unsatisfactory. (115) Hollander refers to a series of uneventful pregnancies in women receiving gold (147) and Miyamoto, et al. report on 26 patients who received gold throughout pregnancy and 43 who discontinued chrysotherapy in early to mid pregnancy. (148) All children were normal at delivery with the exception of two from the last group who had minor hip anomalies.

The two cases described here confirm that gold passes from maternal to the foetal circulation at amounts which are comparable, but not in excess of, those achieved during conventional chrysotherapy. Accumulating evidence suggests that gold therapy in pregnancy can be safely continued in those patients whose disease is sufficiently severe that discontinuation would be unacceptable. However, the absence of any long-term follow-up of offspring
exposed to gold in utero and the suspicion from the Japanese work and the fatal case reported here that toxicity may occur suggest that it is prudent to avoid chrysotherapy in pregnancy wherever possible.

Laboratory Investigations in Gold-Induced Thrombocytopenia

Case 1 (Mrs. E.A.) This lady entered hospital with a rash, renal damage and thrombocytopenia three months after commencing treatment with gold. She was treated with Dimercaprol, initially by intramuscular injection, but because of severe bruising this was changed to an intravenous infusion in an intra-lipid solution. Transfusions of blood and platelets were also given.

The patient remained in hospital for the next eight months except for one period of four days when she was at home. Her condition fluctuated considerably with periods when she had bruising, haematuria, melena and epistasis alternating with improvement. There were few problems with infection although a klebsiella septicaemia developed on one occasion. Five months after admission she became very drowsy and an intra-cranial haemorrhage was suspected but her condition improved without specific treatment. In March 1980, eight months after the onset of her symptoms she again became drowsy, disorientated, then unconscious and she fell from the bed. Her condition rapidly deteriorated and ten hours later she died.

The post mortem examination showed that she had suffered a large right cerebral haemorrhage. In addition there were signs of haemorrhage beneath the skin, in the mouth and throat, trachea and lungs, heart and urinary tract. The course of her disease is summarised in Fig. 2.8. A fall in serum gold concentration was quickly achieved with Dimercaprol and there was some increase in the number of circulating platelets during the first period of chelation treatment. However in view of the subsequent failure for the bone marrow to recover blastic function this temporary response could have been a steroid induced release of semi-mature platelets. Thereafter the platelet count fluctuated between 2 and $30 \times 10^9/\text{L}$ and multiple transfusions of blood and platelets were required to maintain even this level. Serum gold concentrations fell during chelation therapy and then slowly increased to about 2.54 $\mu\text{mol/L}$. At later periods concentrations fluctuated considerably which may have been consequent upon the clinical condition although insufficient measurements were made
to allow a reliable comparison. Urinary excretion of gold was never measured. The total amount of Dimercaprol given was 4.9 g.

Case 2 (Mrs. J.G.) This lady also entered hospital after the onset of a widespread rash a few months after starting chrysotherapy. At this point she had received 450 mg sodium aurothiomalate and regular blood and urine tests had revealed no sign which was predictive of toxicity. There was no eosinophilia nor neutropenia. The dermatitis responded to topical application of steroid but haematological tests after four days revealed the development of a pancytopenia which was confirmed by bone marrow examination. Treatment with oxymethalone was commenced and she was discharged home because of the risk of catching a hospital infection.

A few days later she complained of generalised malaise and vomited blood and was readmitted to hospital. Reverse barrier nursing was commenced and vigorous antibiotic therapy was instituted to reduce bowel flora. She required daily transfusions of platelets and blood but her condition continued to decline. Chelation therapy with intramuscular Dimercaprol was begun the day after admission and maintained for two weeks. Three weeks after admission, bleeding occurred from the gums and palate which did not respond to local treatment. Two days later she deteriorated rapidly and died. Blood cultures taken a few hours before death showed a coliform and Staph. albans septicaemia and post mortem examination revealed ulceration and haemorrhage in the pharynx, oesophagus and other sites also.

As is shown in Fig. 2.9 treatment with oxymethalone failed to produce any response by the bone marrow and her platelet count was maintained at 10–20 x 10⁹/µl only by daily transfusions of blood and platelets. She received 2.2 g Dimercaprol to chelate and excrete the gold. The pre-chelation serum gold concentration was 2.08 µmol/µl and after ten days' treatment the level had fallen to 1.17 µmol/µl. Urinary gold excretion during the first day of treatment was 3.51 µmol and after ten days the removal rate had increased to 4.43 µmol. The day before her death both the serum and urine gold levels had increased to 2.44 µmol/µl and 8.88 µmol/24 hours respectively even though she had not received chelating agent for two weeks.
Fig. 2.8 Summary of clinical, therapeutic and investigative parameters in a patient with thrombocytopenia (Mrs. A.E.).
Fig. 2.9  Summary of clinical, therapeutic and investigative parameters in a patient with thrombocytopenia (Mrs. J.G.).

BAL (mg/day)

Gold (μmol/L) 2

Platelets (x10⁹)

Sodium aurothiomalate (mg)

Clinical milestones

Comence gold

Blisters on fingers

Rash, pruritis, mouth ulcers

in hosp

in hospital

died

MONTHS
Gold induced thrombocytopenia is not a very common development in chrysotherapy and these two cases illustrate that there is no accepted form for its treatment. While removal of gold by chelating agents is usually attempted, a standard regime of dose, duration etc. has not been defined. The chelating agent generally used is Dimercaprol, 200 mg per day given as divided doses by intramuscular injection, although with the first patient the Dimercaprol was also given as an intravenous infusion with intra-lipid. This technique has apparently only been used on one previous occasion and would appear to be effective in maintaining low concentrations of serum gold.

Treatment was commenced soon after the symptoms appeared in the first case whereas with Mrs. J.G. chelation was not started until two months after the first symptoms and three weeks after entry into hospital.

In neither case was there any attempt to relate chelation to the amount of gold given, the serum gold concentrations or to the patient's clinical condition. Platelet production in the bone marrow however remained virtually unchanged whatever the intensity of therapy or the concentration of gold in serum. England and Smith suggested that the total dose of Dimercaprol (in grams) to be administered should be thirty times the total dose of gold (in grams) and described one case in which this regime had proven to be successful in stimulating a bone marrow response. (149) In these two cases only 50% and 30% of this recommended amount was given respectively.

The serum, urine and tissue gold results (Figs. 2.8 and 2.9 and Table 2.17) measured on samples taken near to or after death demonstrated that the chelation therapy used had failed to remove considerable proportions of the body burdens of gold. Whether a more determined course of treatment with Dimercaprol as proposed by England and Smith (149) would have proved successful remains speculative. The presence of the residual gold would however have been realised if urine rather than serum gold concentrations had been monitored. Possibly therefore a regime of more aggressive chelation, continued until little further excretion occurs, should be considered if such situations arise again.

Demonstration of a persistent body burden of gold is consistent with the compartmental model proposed by Gerber et al. (84) The shallow compartment (including the serum) could represent the tissues containing
available gold while the deeper compartment (? includes the bone marrow) and possibly less available to Dimercaprol, would have a very much slower rate of clearance. At the same time however it is not apparent whether, even if gold were removed from the bone marrow, the activity of the tissue would be readily restored.
CHAPTER THREE

STUDIES WITH RADIOLABELLED COMPOUNDS
3.1 INTRODUCTION

From the discussion in the last two chapters it is apparent that previous attempts to determine a detailed appraisal of how gold compounds behave when introduced into the body and how they operate in conditions such as rheumatoid arthritis, have been concerned only with the gold. The associated molecules - thiomalate, thioglucose etc. have been ignored, overlooked or assumed to be irrelevant.

It is now accepted by almost all authorities that serum gold levels are not related to response during chrysotherapy. Therefore in seeking parameters which might provide a reliable guide, further features of the metabolism of these drugs require to be determined. The chemistry of gold compounds (Chapter 1) suggests that thiol complexes will be of considerable importance in *in vivo* reactions, particularly in the formation of mixed thiol species and in thiol exchanges. The actions of the associated molecule might therefore be expected to be not merely as a therapeutically inert transporter but also to influence absorption and disposition of gold and hence, ultimately, the patient's response.

In view of the enigma concerning serum gold concentrations and the suspected importance of the associated molecule vis a vis therapeutic response and gold pharmacology, it was felt appropriate to investigate the metabolism of the thiomalate portion of the molecule together with that of gold.

In seeking to accomplish this goal, it was considered essential to obtain sodium aurothiomalate with the thiomalate moiety radioactively labelled. Such material was not available commercially and the first part of this work therefore involved the radiochemical synthesis of sodium(1,4-\(^{14}\)C)aurothiomalate and (1,4-\(^{14}\)C)thiomalic acid. The preparation of these compounds allowed the investigation of disposition, excretion and intracellular distribution of gold and radioactive carbon and also *in vitro* experimental work on the binding of gold thiomalate to protein.

3.2 RADIOCHEMICAL SYNTHESSES

Synthesis of carbon-14 labelled thiomalic acid was based upon reactions described by Holmberg and Schjanberg. (150) These authors prepared acetylthiomalic acid from maleic acid and thioacetic acid. Removal of the acetyl group was then effected by sodium hydroxide hydrolysis and, upon acidification, the thiomalic acid precipitated from solution. Since carbon-14
labelled maleic acid is not available (1,4-\textsuperscript{14}C)maleic anhydride was used to prepare (1,4-\textsuperscript{14}C)acetylthiomalic anhydride which was then hydrolysed to give (1,4-\textsuperscript{14}C)thiomalic acid (Fig. 3.1). Gold(1) was added from gold iodide on to the sulphur atom of (1,4-\textsuperscript{14}C)thiomalic acid and the sodium salt precipitated from solution by the addition of sodium hydroxide and ethanol.

3.2.1 Materials and Methods

1. Reagents: (1,4-\textsuperscript{14}C)maleic anhydride, specific activity 20 mCi/ mmol. Purchased from Amersham International. Maleic anhydride, thioacetic acid and mercaptosuccinic acid (thiomalic acid) were obtained from Sigma Chemical Co. Scintillant reagent: 0.55% 2,5-diphenyloxazole in Synperonic NXP (I.C.I. Ltd., Billingham, Cleveland)/toluene (1:2 v/v). Other chemicals were obtained from B.D.H. Ltd.

2. Extraction apparatus. The system shown in Fig. 3.2 was used for continuous extraction into hot solvent.

3. Thin layer chromatography. Samples were applied to pre-prepared silica gel G1500 thin layer chromatography plates. Three different solvent mixtures were used; I methylisobutyl ketone: formic acid: water (40:20:20). II water: ethanol (1:9). III butanol: acetone: acetic acid: water (70:70:20:40). The positions of separated compounds were visualised by spraying the plates with 1% w/v potassium permanganate.

4. Measurement of radioactivity. Accurately weighed samples of the radioactive products were dissolved in distilled water. Duplicate 10 \textmu l aliquots from each solution were added to scintillation vials containing 4.0 ml scintillant reagent and 0.5 ml distilled water. The activities were measured in a Packard 2425 liquid scintillation counter. Counting efficiencies were determined by internal standardisation using 10 \textmu l (\textsuperscript{14}C)hexadecane of known activity and the radioactivity, disintegrations per minute (dpm), calculated from the formula:

\[ \text{dpm}.t = \frac{\text{cpm}.t}{\text{cpm}.th - \text{cpm}.t} \times \text{dpm}.h \quad \text{where} \quad t = \text{test sample} \]

\[ h = \text{hexadecane} \]

\[ th = \text{test sample + hexadecane} \]

Radioactivity in curies was then calculated from, \( 2,200 \times 10^9 \text{ dpm} = 1 \text{ Ci} \).
Fig. 3.1 Synthesis of $^{14}$C-thiomalic acid from $^{14}$C-maleic anhydride and thioacetic acid.

\[
\begin{align*}
&\text{CH}_3\text{-C-SH} + \text{HC}^{14}\text{C} \xrightarrow{4\text{NaOH}} \text{HS}-\text{CH} + \text{CH}_3\text{-C-ONa} + 2\text{H}_2\text{O} \\
&\text{CH}_3\text{-C-S-CH}^{14}\text{C} \xrightarrow{\text{2Na}_2\text{SO}_4} 2\text{HS-CH} + 3\text{Na}_2\text{SO}_4
\end{align*}
\]
5. Measurement of gold. Gold containing compounds were accurately weighed and dissolved in distilled water. A series of dilutions were prepared from this initial solution together with standard gold solutions having concentrations in the range of 0.5 to 10.0 µg/ml. Standard and sample solutions were aspirated into an atomic absorption spectrophotometer (operating conditions as in Table 2.2) and the absorbance readings noted. The concentration of gold in the sample solutions were calculated by reference to the standards and from these concentrations, the amount of gold in the analyte determined.

6. Nuclear Magnetic Resonance (NMR). Samples for NMR analysis were dissolved in deuterated acetone and the spectra scanned using a Perkin Elmer R24A high resolution NMR spectrometer.

7. Infra Red Analysis. Infra red spectra of samples were prepared on a Perkin Elmer 577 spectrometer.

3.2.2 Procedures

1. Preparation of (1,4-\textsuperscript{14}C)thiomalic acid.

1 mCi of (1,4-\textsuperscript{14}C)maleic anhydride (specific activity 20 mCi/mmol) was supplied in four vials each containing 250 µCi. The contents of each vial were dissolved in approximately 0.2 ml dichloromethane and the combined solutions added to 98 mg (1 mmol) non-radioactive maleic anhydride in a weighed glass tube (100x8 mm, 3.0 ml capacity). The dichloromethane was gently evaporated under a stream of nitrogen. Cooling in an ice-bath was essential to avoid loss of maleic anhydride by volatilization (see below).

Thioacetic acid, 0.1 ml, was added slowly over approximately 30 seconds to the maleic anhydride, at 0°C. Air in the tube was displaced by nitrogen, the tube stoppered and gently mixed until the contents formed a milky suspension which went slowly into solution. After standing overnight at 4°C a white crystalline mass with no supernatant fluid was present. The tube was heated in a water bath until the mass melted (71-74°C) and was then slowly cooled to 4°C. Recrystallization occurred as the temperature fell. The solid material was dissolved in approximately 1 ml benzene warmed to no more than 60°C, allowed to recrystallise slowly and left overnight at 4°C under nitrogen. The pale yellow supernatant was decanted and the residue washed with a further 1 ml benzene. The crystals were
dried by a stream of nitrogen and the tube reweighed. The yield of acetylthiomalic anhydride was 145 mg (83%).

Sodium hydroxide (4.0 mmol) dissolved in 1.5 ml water was added to the tube, the air displaced by nitrogen and solution of the contents effected by repeated inversion. The mixture was left at 4°C overnight and excess sulphuric acid (2.5 mmol in 1.5 ml water) was added. The acidified contents were transferred quantitatively to the continuous extraction apparatus (Fig. 3.2) and extracted with 75 ml ethylacetate. The optimum time for extraction was determined in preliminary experiments by removing a few drops of ethylacetate or the aqueous sample solution and adding to tubes containing 2% w/v ferric chloride, which forms a blue colour in the presence of thiol groups. Extraction for 45 minutes was found to be appropriate.

The extract was evaporated to dryness under nitrogen to yield 124 mg (83%) of white thiomalic acid, m.pt 149°C (Sigma thiomalic acid, 145-146°C).

Thin layer chromatography using solvent system I revealed a single spot with an Rf of 84.4. Examination of the chromatogram using an isotope scanner showed a concentration of radioactivity at the position having the same Rf value. Thiomalic acid from Sigma had an Rf value of 83.3 on the same chromatogram. The radioactive content of the prepared thiomalic acid was 105 µCi with a specific activity therefore of 4.88 µCi/mg or 732 µCi/mmol.

Comparisons of NMR and infra red spectra of prepared and commercial thiomalic acid indicated that traces of an acetyl group (probably acetyl-thiomalic anhydride) were present following prolonged extraction into ethylacetate. Residues in these situations were waxy rather than powdery. With careful timing of the extraction this contamination was avoided and the eventual NMR and infra red scans of synthesised and commercial thiomalic acid were identical.

The specific activity of thiomalic acid was disappointingly low and since the yield (83%) was good considerable loss of radioactivity obviously occurred. Large amounts of carbon-14 were not detected in any of the residual solutions. It was therefore suspected and confirmed by experiment and communication with Amersham International that small amounts of maleic anhydride will readily volatilize from dichloromethane.
Fig. 3.2 Apparatus used for extraction of thiomalic acid into ethyl acetate.
2. Preparation of sodium(1,4-\textsuperscript{14}C)aurothiomalate.

Two techniques were investigated for the addition of gold on to the thiol group of thiomalic acid. One used aurous cyanide and the second aurous iodide to provide the gold(I) atom.

Aurous cyanide: The incorporation of gold into thiomalic acid from aurous cyanide was described by Trenner and Bacher (151) (Fig. 3.3). Gold potassium cyanide, 0.288 g, was dissolved in 5.0 ml distilled water and 1.0 ml of 10\% v/v hydrochloric acid was added. The solution was heated in an evaporating dish to about 50\degree C whereupon yellow aurous cyanide precipitated. The dish was placed on a steam bath and the aqueous solution evaporated to dryness with the simultaneous loss of hydrogen cyanide. The residue was suspended in water, filtered, re-washed with water and finally dried over P\textsubscript{2}O\textsubscript{5}.

Aurous cyanide 56 mg (0.25 mmol) was suspended in 20 ml distilled water and 104 mg thiomalic acid (0.69 mmol) dissolved in 32 ml distilled water was added. The mixture was heated on a steam bath for 30 minutes and most of the suspended matter dissolved. When the solution cooled the undissolved solids were removed by filtration and the filtrate evaporated to dryness on the steam bath. The residue thus formed was triturated four times with hot ethylacetate to remove unreacted thiomalic acid and dried overnight in a vacuum oven at 100\degree C.

Since this procedure required 2.75 moles of thiomalic acid per mole of gold and the yield was poor (about 10\%) it was not used and the alternative procedure with aurous iodide was adopted.

Aurous iodide: Details of this method was supplied by Amersham International where it is used during the preparation of sodium(\textsuperscript{195}Au)aurothiomalate (Fig. 3.4).

Gold metal was dissolved in aqua regia and diluted to a concentration of 20 mg/ml. 1 ml of this solution was evaporated just to dryness and the residue dissolved in 1 ml concentrated hydrochloric acid. 9.5 mg potassium chloride was added, mixed and the solution quantitatively transferred to a weighed test tube (100x8 mm). Potassium iodide (49.8 mg in 1 ml water) was added producing a brown, flocculent precipitate. Water was added almost to fill the tube and nitrogen blown in to displace the residual air. The tube was stoppered, the contents mixed and
**Fig. 3.3** Preparation of aurothiomalic acid using aurous cyanide as the gold(1) donor.

\[
K(Au(CN)_2) + HCl \rightarrow AuCN + HCN + KCl
\]

\[
\begin{array}{c}
\text{COOH} \\
\mid \\
\text{CH}_2 \\
\mid \\
\text{CHSH} + \text{AuCN} \rightarrow \text{CHSAu} + \text{HCN} \\
\mid \\
\text{COOH}
\end{array}
\]

**Fig. 3.4** Preparation of sodium aurothiomalate using aurous iodide as the gold(1) donor.

\[
\begin{array}{c}
\text{Au} + HCl \rightarrow \text{AuHCl}_4 \\
\end{array}
\]

\[
\begin{array}{c}
\text{AuHCl}_4 + \text{KCl} \rightarrow \text{AuKCl}_4 + \text{HCl} \\
\end{array}
\]

\[
\begin{array}{c}
\text{AuKCl}_4 + 3\text{KI} \rightarrow \text{AuI} + 4\text{KCl} + \text{I}_2 \\
\end{array}
\]

\[
\begin{array}{c}
\text{COOH} \\
\mid \\
\text{CH}_2 + \text{AuI} + 3\text{NaOH} \rightarrow \text{CH}_2 + \text{NaI} + 3\text{H}_2\text{O} \\
\mid \\
\text{CHSH} \\
\mid \\
\text{COOH}
\end{array}
\]
centrifuged. After removal of the supernatant, the precipitate was washed with water and twice with ethanol. The precipitate was dried under a stream of nitrogen, in the dark, and the tube reweighed. 32 mg pale yellow gold(I) iodide was thus prepared.

(1,4-$^{14}$C)thiomalic acid, 15 mg, was dissolved in 1 ml of water and added to the gold iodide forming a yellow suspension. 4M sodium hydroxide was added dropwise until the gold iodide just dissolved. The solution was poured into a weighed 50 ml tube and about 40 ml ethanol, saturated with sodium acetate, added. After standing overnight, the tube was centrifuged and the supernatant removed. The precipitate was washed with ethanol, dried under nitrogen and weighed.

The synthesised sodium aurothiomalate had a gold content of 48.9 μmol (from an initial 100 μmol) while the radioactivity was 36.5 μCi (amount added = 73.2 μCi). The yields of gold and carbon-14 were 48.9% and 49.9% respectively. Thus the specific activity, 746 μCi/mmol was similar to that of the thiomalic acid. Thin layer chromatography using solvent systems II and III revealed a compound containing gold and carbon-14 which had Rf values of 100 and 46.5 respectively. These were similar to the Rf values found with sodium aurothiomalate obtained from May & Baker Ltd. (100 and 47.5) and quite distinct from those of thiomalic acid (0 and 84 respectively).

2.3 EXPERIMENTS WITH RADIOLABELLED COMPOUNDS

3.3.1 Introduction

Despite the poor radioactive yield achieved in the synthesis of (1,4-$^{14}$C)thiomalic acid the intermediate and final products were prepared in sufficient amounts and with the necessary radiochemical purity for use in the following series of experiments.

1. Disposition and excretion of gold and carbon-14.

From the work with patients with rheumatoid arthritis and from earlier animal experiments the fate of gold after an injection of sodium aurothiomalate is reasonably well understood. The rate of absorption, transport in the blood, uptake into tissues and excretion into the urine have been repeatedly examined and were described in Chapter 1. It has also been demonstrated that it is the gold which is the effective agent in rheumatoid arthritis and that the therapeutic response afforded by myo-
chrisin is not mimicked by thiomalate whereas other gold complexes e.g. aurothioglucose are also effective anti-rheumatic drugs.

The thiomalate fraction of the drug has therefore been assumed to be unimportant once the injection has been given and that its only role is to provide a stable, soluble form of gold(I). The assumption most often voiced is that gold will readily dissociate from thiomalate in vivo, attach to albumin and be transported throughout the body while the residual part of the drug will probably be metabolised (possibly via the tricarboxylic acid cycle with expiration as CO₂), and have no further influence upon the activity of the gold.

By using sodium(¹⁴C)aurothiomalate and (¹⁴C)thiomalic acid both fractions of the injected compound can be followed and the validity of these assumptions may be checked.

2. Subcellular distribution of gold and carbon-14.

The fate of gold following its entry into the cell has been examined by Danpure and his co-workers (152,153) using sodium(¹⁹⁸Au)aurothiomalate. These authors suggested that the metal initially goes into the cytosol but is then transferred into lysosomes which may contain up to 90% of hepatocellular gold. This concentration of gold in lysosomes is in agreement with the morphological studies of several others (99,122,154-157) and the accumulation within these organelles can be so exaggerated as to earn them the descriptive pseudonym "aurosomes".

Recent interest in the metabolism of metals has realised the importance of the low molecular weight proteins, the metallothioneins (158). Synthesis of these proteins is induced by a primary exposure to a metal and with additional exposure accumulation takes place in those tissues rich in the protein. The thionein-rich tissues are the liver and kidney although the intestine, spleen and probably other organs also contain these proteins. While the thionein binds the metal responsible for its induction certain other cations can also be incorporated. These proteins have been repeatedly isolated as a consequence of exposure to cadmium, zinc, copper and mercury and interactions between these metals and the proteins have been described but there are also reports of thionein synthesis following exposure to other metals including gold.
Metallothioneins are localised within the cytoplasm and therefore at least two features to the intracellular metabolism of gold must be considered i.e. (a) incorporation into cytosolic, low molecular weight binding protein and (b) uptake into the lysosomal vesicles. Whether these features are independent or whether gold taken into tissues will pass from one system on to the second does not appear to have been previously considered.

Additional to these two mechanisms is the relevance or otherwise of the associated carrier molecule. It has been demonstrated (Fig. 3.10) that radioactive carbon enters cells when rats are given sodium(1,4-$^{14}$C)aurothiomalate although it is not apparent whether this takes place before or after separation from gold. If intact drug does enter the cell it is relevant to enquire how long the gold and thiomalate remain together or do they immediately proceed along separate metabolic paths?

To examine these questions the intracellular fate of gold and radioactive carbon was followed in rats after the administration of sodium (1,4-$^{14}$C)aurothiomalate and (1,4-$^{14}$C)thiomalic acid.


The normal physiological route of excretion for most metals to which man or animals are exposed is via the faeces and with few exceptions urinary elimination is quantitatively much less important. However, with most metals the major proportion of the faecal burden is derived from ingested metal which has remained unabsorbed throughout the passage down the gut. That fraction of the ingested metal which is assimilated can then be excreted into the intestine and enter the faeces either predominantly through the bile e.g. copper or through intestinal secretions, e.g. zinc, or through both of these routes, e.g. manganese. (159)

Since sodium aurothiomalate is administered by intramuscular injection, faecal gold can only be derived from that which is subsequently secreted into the alimentary tract. The work with human volunteers and with animals (Chapter 1) and the results of the disposition and excretion experiments reported in Section 3.3.3 have shown that urinary excretion of gold is of greater significance during the first 24 hours after an injection but that during the following days faecal elimination probably assumes a greater importance.
Transfer of compounds into the bile from the liver cell may involve a process of passive diffusion as with albumin or occur against a concentration gradient and therefore require expenditure of cellular energy. More than one mechanism for the transport of components into the bile have been described. In addition to hepato-biliary membrane transport of small molecular weight compounds such as bilirubin, lysosomes and smaller endocytic vesicles convey and release their contents into the canaliculi by exocytosis. (160)

Transfer of IgG, and IgM and immune complexes from the liver to the bile is accomplished by lysosomes. The experimental work examining the intra-cellular distribution of gold demonstrated the importance of lysosomes in the hepatic metabolism of gold (section 3.3.3). It might be expected therefore, that given this concentration of gold in the lysosomes, the known association between gold and immune complexes in inflammatory cells and fluids and the excretion of gold in the faeces, that gold would be discharged from lysosomes into the bile. The extent of any entero-hepatic circulation upon the passage of gold thence into the faeces is unknown but Kapelowitz et al. (102) were able to collect biliary drainage in a gold-treated patient and these authors concluded that less than 50% of faecal gold was derived from the bile.

Other experiments reported in this Chapter give no information relevant to the movement of thiomalate from the liver, therefore by giving rats the radioactively labelled thiomalic acid and sodium aurothiomalate and monitoring the appearance of gold and carbon-14 in the bile it should be possible to assess the importance of biliary excretion and, by comparison with the excreted compounds, to make an estimate of entero-hepatic circulation.


The distribution of gold within the plasma was discussed in Chapter 1. All reported studies have demonstrated that very large proportions of the circulating gold are bound to albumin with the proportion of non-albumin bound metal found to be between 0 and 25%. Very little attention however has been focussed upon efforts to resolve questions concerning the nature of the albumin-gold complex.

On the basis of reactions involving albumin, sodium aurothiomalate and other thiol compounds, and thiol-blocking agents, Gerber (8) and Danpure (93,161) propose that the interaction is between a cysteinyi residue on
the protein and gold(l). Other workers (162,163), while confirming a major binding site with a high affinity constant ($6.1 \times 10^3 \text{M}^{-1}$ and $3.3 \times 10^4 \text{M}^{-1}$) also detected at least three other binding sites with much lower affinities.

Gerber (8) added sodium aurothiomalate to albumin and attempted to assay free thiol groups as a ferricyanide reducing agent. He was unable to demonstrate release of thiomalic acid when the protein-drug complex was formed. Mason (162) and Jellum et al. (164) also sought to determine whether thiomalate was produced as the complex was formed and both groups concluded that there was some release.

The results of Danpure (161) showed both release and retention of thiomalate in very simple in vitro incubation systems. As a primary model he examined the interaction between sodium aurothiomalate and cysteine and subsequently took the gold compound and albumin. In the first experiments thiomalic acid was evolved as the two components reacted but with the protein present he was unable to detect the presence of free thiol groups.

Thus the nature of the major circulating fraction of gold in the blood is only partially understood and realistic descriptions of the further metabolism of the gold compound can only be made when the composition of the complex is determined. It would be expected that in vivo, the presence of thiomalate in the albumin-gold complex might influence further thiol exchange reactions which have been predicted from the chemistry of gold(l). Thiomalate therefore could influence or modulate the availability or movement of gold into other plasma fractions or into tissues. Since this point is of such importance to the metabolism of gold and the mechanism of its action, the binding of gold and carbon-14 to albumin was examined using the radioactive thiomalic acid and sodium aurothiomalate compounds.

3.3.2 Materials and Methods

Experimental

1. Disposition and excretion of gold and carbon-14.

Groups of three male Wistar Albino rats weighing about 200 g were injected with either (1,4-$^{14}$C)thiomalic acid, 5.3 mg/Kg, or sodium (1,4-$^{14}$C)aurothiomalate, 8.2 mg/Kg, into the thigh muscle and then maintained in glass metabolic cages (Fig. 3.5) for 72 hours. Air was
Fig. 3.5  Glass metabolism cage used for metabolic experiments with (1,4-\textsuperscript{14}C)thiomalic acid and sodium(1,4-\textsuperscript{14}C)aurothiomalate in rats.
drawn through the apparatus by a suction pump, first through soda lime in order to absorb the carbon dioxide, then to the animal and finally through three liquid traps which collected the expired carbon dioxide. The three traps were collected in series, each containing 200 ml ethanolamine in ethyl digol (12% v/v) and maintained at 0°C. The trapping medium was changed at 90 minutes and at 3, 6, 24, 48 and 72 hours. Urine and faeces were collected over three successive periods; 0-24 hours, 24-48 hours, 48-72 hours. Blood was collected at 72 hours by cardiac puncture with the animals under barbiturate anaesthesia. The animals were then killed and tissues removed for analysis.

To assess the transfer of radioactive compound from the site of injection in the muscle into the circulation a further experiment was conducted. Rats were injected with either sodium\(1,4^\text{14}\)aurothiomalate or the \(1,4^\text{14}\)thiomalic acid as previously described but the site of injection on the thigh was marked. Twenty-four hours later the animals were killed by cervical dislocation. The area of skin approximately one centimeter square immediately above the point of injection was removed and the underlying muscle dissected away and weighed. The sample of muscle was prepared for analysis of radioactivity by solubilization, as described below.

2. Subcellular distribution of gold and carbon-14.

(1) Male, Wistar Albino rats were injected sub-cutaneously with either sodium\(1,4^\text{14}\)aurothiomalate, 10.8 μmol/Kg (1.91 μCi) or \(1,4^\text{14}\)thiomalic acid, 27.7 μmol/Kg (2.03 μCi). The doses were selected to provide approximately 2 μCi radioactivity per experiment. At 2 hours and 24 hours after injection five rats from each group were killed by cervical dislocation. The abdomen of each rat was opened to expose the liver which was removed and washed with ice cold buffered isotonic sucrose solution. The liver was homogenised and the cellular organelles separated by differential centrifugation. Each fraction was analysed for radioactivity and for gold and the purity of the separations were assessed by measurement of marker enzyme activities.

(2) Male, Wistar Albino rats were injected sub-cutaneously with sodium \(1,4^\text{14}\)aurothiomalate and killed two hours later by cervical dislocation. The livers were removed, homogenised and centrifuged to obtain a mixed mitochondrial-lysosomal preparation. This fraction was further separated
by sucrose density gradient centrifugation. The subcellular material removed at intervals along the gradient was also analysed for radioactivity, gold and enzyme activities.

Homogenisation and Differential Centrifugation.

Excised liver was washed with ice-cold sucrose (0.25 M sucrose buffered in 5 mM Tris, pH 7.4), blotted dry and weighed in a tared beaker containing 20 ml cold sucrose. The liver was cut into small pieces no larger than 0.5 cm cubes and placed in a pre-cooled Potter-Elvejhem tube. Homogenisation was effected by three complete strokes of the teflon pestle at 2,400 r.p.m. Further buffered sucrose solution was added to adjust to 1 g liver per 10 ml. The suspension was sieved into a cold beaker and then poured into 30 ml centrifuge tubes. The entire procedure, from killing the rats to commencement of centrifugation was carried out with a minimum of delay and was completed within five minutes. All solutions and equipment were pre-cooled in an ice-bath before use.

The homogenate was centrifuged at 600 g for 10 minutes and the supernatant poured into a cold beaker. The pellet was resuspended in buffered sucrose solution, recentrifuged and the two supernatants combined. The sediment, representing a crude nuclear fraction together with large cell debris and non-ruptured cells was labelled 'N'. The combined supernatants were poured into tubes and centrifuged at 12,000 g for 15 minutes using an 8x50 ml angle rotor on an MSE High Speed 18 centrifuge. The supernatant was removed, the pellet resuspended in buffered sucrose solution and the contents recentrifuged. The supernatant was again removed to leave the mitochondrial-lysosomal fraction which was labelled 'M+L'. The two supernatants were combined for a further centrifugation step at 120,000 g for 60 minutes in an 8x35 ml angle rotor using an MSE Super Speed 65 centrifuge. The precipitate, represented the microsomal fraction ('M') and the supernatant, the cytosol.

The pellets were resuspended in sucrose solution to a final volume of 5 ml and the volume of the residual cytosol was measured. These samples were divided into aliquots and stored at -20°C.

Sucrose Density Gradient Centrifugation.

Buffered sucrose solutions were prepared at concentrations of 0.25 M, 0.3 M, 1.6 M and 2.0 M sucrose in 5 mM Tris, pH 7.4.
In three 20 ml centrifuge tubes, sucrose density columns were prepared using a gradient mixer to give linearly decreasing density columns with 2.0 M sucrose at the base and 0.25 M sucrose at the top. The columns were kept at 0°C for about two hours before use.

Liver homogenates were prepared as described above and centrifuged to remove the nuclear material. The supernatants were centrifuged once at 12,000 g to isolate a crude mitochondrial-lysosomal fraction which was resuspended in 0.25 M buffered sucrose solution. Using a 2.0 ml syringe, 1.5 ml of each M + L suspension was carefully layered on to the top of a sucrose gradient and placed into a 3x25 ml swing-out rotor. Centrifugation at 9,000 g was carried out for 30 minutes to separate the elements of the suspension.

A 50 ml syringe containing 2.0 M buffered sucrose solution was mounted with the piston connected to a slow drive motor. The output from the syringe led to a narrow tube inserted down the centre of the sucrose column. The motor was calibrated to expel buffered sucrose solution from the syringe at 4 ml per minute. The buffered sucrose solution pumped into the base of the column gradually displaced the contents from the top. Displaced solution containing separated sample was collected in aliquots of 1.0 ml and twenty were obtained from each column.


Male, Wistar Albino rats weighing about 200 g were treated with either (1,4-14C)thiomalic acid, 27.7 μmol/Kg (2.03 μCi) or sodium (1,4-14C)aurothiomalates 10.8 μmol/Kg (1.91 μCi) in saline. The compounds were pre-administered by sub-cutaneous injection either two or twenty four hours before commencement of bile collection or by intravenous injection as soon as bile cannulation was achieved and bile flow established. In some experiments unlabelled sodium aurothiomalate was given.

Bile Duct Cannulation.

The rats were anaesthetised with sodium pentobarbitone (Sagatal) and the bile duct exposed by mid-line incision and displacement of first the skin and then the abdominal wall. Polythene tube 0.8 mm internal diameter was inserted into the bile duct through a hole cut in the wall of the vessel about 1 cm distal to the junction of the hepatic ducts. The cannula was retained in position by two ligatures. To prevent dehydration of the animal the cut edges of the abdominal wall were stitched together and
the skin incision closed with clips. The bile duct cannula emerged between the incision in the abdominal wall and through a small hole pierced in the skin.

To allow intravenous administrations the jugular veins were exposed by lateral incisions in the neck. The larger vein was supported from beneath while the injection was made. The skin was reclosed with clips.

A rectal thermometer was used to monitor body temperature which was maintained at 35-36°C by placing the rat on a heated blanket. Careful control of body temperature was essential to avoid thermal related changes in biliary composition. Additional administration of Sagatal was provided intra-peritoneally as necessary to maintain anaesthesia. Bile was collected into pre-weighed, metal-free polypropylene tubes and blood was collected into heparinised tubes by sectioning the tail vein or by cardiac puncture at the termination of the experiments.

4. *In vitro* protein binding.

(a) Binding of (1,4-\(^{14}\)C)thiomalic acid to albumin.

To 10 ml of albumin solution (4% w/v, Bovine fraction V albumin, Sigma, London, in 0.066 M phosphate buffer pH7.4 containing 0.9% w/v sodium chloride and 0.005% w/v sodium azide (hereafter called phosphate buffer)), 54.0 μg thiomalic acid and 50 nCi (1,4-\(^{14}\)C)thiomalic acid were added in a volume of 10 μl. The solution was mixed and stood at room temperature for 30 minutes. 1.5 ml was removed and separated by gel filtration on a 20x250 mm column containing Sephadex G75 (Pharmacia, London) at a flow rate of 4 ml per hour. Fractions of the eluate were collected every 30 minutes for 20 hours. A further 1.5 ml was similarly separated after an incubation period of 24 hours.

(b) Binding of sodium(1,4-\(^{14}\)C)aurothiomalate to albumin.

(i) To 10 ml of 4% albumin solution in phosphate buffer, 142.9 μg sodium aurothiomalate and 50 nCi sodium(1,4-\(^{14}\)C)aurothiomalate were added in a volume of 10 μl. The solution was mixed and incubated at room temperature. 1.5 ml was removed and separated by gel filtration as described above after incubation periods of 0, 5, 15 and 30 minutes, 24 hours and 7 days.
(ii) The gold and radioactive distribution at different molar ratios of sodium aurothiomalate and albumin were investigated. 10 ml of 4% albumin solution in phosphate buffer was taken and an appropriate amount of unlabelled sodium aurothiomalate was added. 50 nCi sodium (1,4-\(^{14}\)C)aurothiomalate was also added, the samples mixed and 1.5 ml removed for separation by gel filtration. The amounts of sodium aurothiomalate added to 10 ml of albumin solution were 14.3 \(\mu\)g, 71.4 \(\mu\)g, 142.7 \(\mu\)g, 285.5 \(\mu\)g, 713.7 \(\mu\)g and 1427.0 \(\mu\)g (i.e. 3.66, 18.3, 36.6, 73.2, 182.9 and 365.9 \(\mu\)mol/l) which gave albumin to drug molar ratios of 161:1, 32:1, 16:1, 8:1, 3.2:1 and 1.6:1 respectively.

(c) Binding of sodium(1,4-\(^{14}\)C)aurothiomalate to Sephadex G75.

1.5 ml phosphate buffer containing 21.4 \(\mu\)g sodium aurothiomalate and 50 nCi sodium(1,4-\(^{14}\)C)aurothiomalate was eluted through the same Sephadex G75 column.

The concentrations of albumin and sodium aurothiomalate used were selected so as to provide approximately the same levels as would be found in a patient during the first hour after an intramuscular injection of sodium aurothiomalate.

Analytical
1. Measurement of radioactivity.

Five scintillation systems were used:

(1) 0.55% 2,5-diphenyloxazole in toluene containing 4% thixotropic gel (Packard, La Grange, IL, USA).

(2) 0.55% 2,5-diphenyloxazole in Synperonic NXP (I.C.I. Ltd.)/toluene (1:2 v/v).

(3) Instagel (Packard).

(4) 0.68% 2(4'-t-butylphenyl)5-(4'-biphenyl)-1,3,4oxadiazole in Triton X 100/toluene (1:2 v/v).

(5) Pico-fluor 30 (Packard).

Samples were treated as follows:

Methanol (4 ml) was added to 5 ml CO\(_2\) trapping medium and the solution counted for radioactivity in 10 ml of scintillant 1.
Urine was diluted as appropriate and 100 µl suspended in 4.0 ml scintillant 2.

Faecal homogenates were prepared in 0.1 M phosphate buffer, pH7.4, to a concentration of 5% (w/v). Samples (500 µl) of the homogenates, were dispensed into glass scintillation vials and heated with 3.0 ml hyamine hydroxide (Packard) at 55-60°C for five hours. After cooling, 30% hydrogen peroxide (250 µl) was added and the solution decolourised overnight. Digests were neutralised with 250 µl 12 M hydrochloric acid and suspended in 10 ml scintillant 3.

Tissue snips (250 mg), were added to 1.0 ml of a mixture of sodium hydroxide (8.0 g), methanol (30 ml), water (60 ml) and Triton X 405 (10 ml of 75% aqueous solution) in glass scintillation vials and heated at 55-60°C for 1 hour. After cooling, 250 µl 30% hydrogen peroxide was added and left overnight. Digests were neutralised with 4 M nitric acid (500 µl) and suspended in 10 ml scintillant 4.

To 500 µl resuspended subcellular fractions prepared from homogenised liver samples, 3 ml hyamine hydroxide was added and the mixture incubated for one hour at 50°C. After cooling, 250 µl 30% hydrogen peroxide was added and the solution decolourised overnight. The samples were neutralised with 250 µl concentrated hydrochloric acid and 10 ml scintillant 3 added.

Samples of separated M + L fractions, plasma and bile (500 µl, 20 µl and 200 µl respectively) were added to 4.0 ml scintillant 5, mixed and left overnight at 4°C and the activity counted.

Samples (0.5 ml) of the fractions eluted from the Sephadex column were added to 4.0 ml scintillant 2, mixed and counted.

Activity was counted using either a Packard 2425 or a Packard Prias scintillation counter. Counting efficiencies were determined by internal standardisation using (14C) hexadecane and radioactivity calculated as described in section 3.2.1.


Tissue and faecal samples requiring destruction of organic material were prepared for analysis using the acid digestion technique (see section 2.3.1).
Gold concentrations were determined by atomic absorption spectroscopy either by aspiration into the flame or, when further sensitivity was required, by electrothermal atomization using the Pye SP9 Video Furnace AAS (Table 3.1).

Biliary gold was measured by pulse sampling atomic absorption spectrophotometry. 100 μl of sample was pipetted into the conical base of an auto-analyser sample cup. The nebuliser capillary of the atomic absorption instrument was inserted into the cup and the sample immediately aspirated completely into the flame. The transient signal was recorded on a rapid-response chart recorder. All analyses were performed in duplicate and excellent precision (less than 5% difference between paired peak heights) was routinely obtained. The peak heights were compared against those obtained from 100 μl of aqueous gold standards similarly aspirated into the flame.

Gold in plasma was measured either by pulse sampling atomic absorption spectrophotometry or by flame aspiration using the method of internal standardization described in section 2.3.2.

Samples collected from the Sephadex column were analysed by flame aspiration and the signals compared with those given by gold standards diluted in phosphate buffer.

3. Thin layer chromatography.

From the samples of urine collected during the first 24 hours of the metabolic experiments, 10 μl were separated on silica-gel G1500 thin layer chromatography plates using butanol, acetone, acetic acid, water (70:70:20:40) as the developing solvent. No pre-treatment to the urine was made. Standard solutions of commercially available thiomalic acid and sodium aurothiomalate were run on the same plate.

Autoradiographs of the developed chromatograms were prepared to identify the radioactive excretory compounds. To prevent powder adhering to the radiograph the chromatography plate was covered by a thin film of cellophane (Clingfilm) stretched over the silica-gel and sealed to the reverse side of the plate. X-ray film, Kodak NS-2T was firmly sandwiched between the silica-gel surface of the TLC plate and a sheet of glass which were then secured together with adhesive tape. The plate was then
Table 3.1 Instrumental conditions used with Pye SP9 Video-Furnace for the determination of gold by electro-thermal atomic absorption spectroscopy.

<table>
<thead>
<tr>
<th>Final temp (°C)</th>
<th>Ramp rate (°C sec(^{-1}))</th>
<th>Time held at temp (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRY phase</td>
<td>120</td>
<td>2</td>
</tr>
<tr>
<td>ASH phase</td>
<td>1000</td>
<td>20</td>
</tr>
<tr>
<td>ATOMISE phase</td>
<td>2500</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>CLEAN phase</td>
<td>3000</td>
<td>&gt;2000</td>
</tr>
</tbody>
</table>

Wavelength - 242.8 nm, band pass - 0.5 nm, lamp current - 8 mA.

During the atomise phase the temperature control and gas stop options were applied. Auto zero was operated at the start of the DRY and ATOMISE phases.
wrapped in at least three layers of light proof paper and placed in a press to ensure no movement of the X-ray film relative to the chromatograph. An exposure period of eight weeks was allowed before the film was developed. Kodak developer and fixer were both diluted, 50 ml + 200 ml water and the films immersed in each solution (developer followed by fixer), for four minutes in each. After washing in running tap water for 3-4 hours the films were suspended until dry. Preparation of the autoradiographs and their development were carried out in a photographic dark room.

After development of the films the TLC plates were sprayed with 0.5% potassium permanganate to determine the position of the thiomalic acid and sodium aurothiomalate markers.

Chromatography and autoradiography of samples of bile was carried out using the same procedures.


The following enzyme markers for subcellular particles were used; succinic dehydrogenase-mitochondria, acid phosphatase-lysosomes, 5’ nucleotidase-membranous material and glucose-6-phosphatase-endoplasmic reticulum (microsomes).

Aliquots of resuspended subcellular fractions and samples of cytosol were allowed to thaw and diluted fortyfold and twentyfold respectively with 0.25 M buffered sucrose solution. The separated M + L fractions were diluted fivefold with 0.25 M buffered sucrose solution.

Protein: Protein concentrations were measured by the method of Lowry using a continuous flow system for analysis. (165)

Succinic dehydrogenase, 5’ nucleotidase and glucose-6-phosphatase were determined using the methods of Pennington (166), Prospero, Burge, Norris et al. (167) and Prospero and Hinton (168) respectively.

Acid phosphatase: A method developed at the Robens Institute, University of Surrey by Hinton and Dobrota was used. Sample (0.5 ml) was added to 0.3 ml 0.3 M sodium acetate buffer, pH5.0 and 0.1 ml 0.016% w/v digitonin. Solutions were warmed to 37°C and the reaction started by the addition of 0.1 ml 1 M βglycerophosphate, pH5.0. The reaction
was terminated after 60 minutes by adding 1.5 ml 6.0% w/v trichloroacetic acid and the tubes were centrifuged to remove precipitated protein. The supernatants were taken for the measurement of released phosphate. All analyses were carried out in duplicate and a substrate blank using 0.25 M sucrose instead of sample was set up. Digitonin was used to release the enzyme from lysosomes and unlike Triton (an alternative releasing agent) does not interfere in the subsequent measurement of phosphate.

Phosphate: The phosphate concentration in samples after enzyme reactions had been completed, were determined by the reaction with ammonium molybdate followed by reduction of the phosphomolybdate complex to molybdenum blue. These reactions were carried out using a continuous flow system for analysis. (169)

Eluate from the Sephadex column was continuously monitored for total protein content by recording the absorbance at 280 nm.

3.3.3 Results

1. Disposition and excretion of gold and carbon-14.

The cumulative excretion of radioactive carbon from the rats injected with (1,4-^{14}C)thiomalic acid is shown in Fig. 3.6. Excretion of carbon-14 via the faeces was relatively trivial and accounted for no more than 2% of the injected dose. Rather more was eliminated by the lungs and about 10% of the administered radioactivity was collected from the expired air, most appearing within the first 3 hours. The major route of excretion was via the kidney with 50% of the injected carbon-14 removed into the urine and as with the CO_2 very little was lost after the first 24 hours.

The pattern of excretion of carbon-14 from the animals given sodium (1,4-^{14}C)aurothiomalate is given in Fig. 3.7. As with the thiomalic acid treated animals, it was the first 24 hours which were important and very little radioactivity was found in samples collected later. Again, as with the rats given (1,4-^{14}C)thiomalic acid, excretion via the faeces was not important while the expired air accounted for slightly more at 12-15% of the given dose. Although renal excretion was again the main route for elimination, a greater proportion of the dose (75%) was removed compared with the (1,4-^{14}C)thiomalate treated rats.
Fig. 3.6 Excretion of carbon-14 in expired air, urine and faeces by rats given (1,4-14C)thiomalic acid by intramuscular injection. Results are expressed as mean ± standard deviation, n = 3.

Fig. 3.7 Excretion of carbon-14 in expired air, urine and faeces by rats given sodium(1,4-14C)aurothiomalate by intramuscular injection. Results are expressed as mean ± standard deviation, n = 3.
The total and urinary excretion of carbon-14 from both experiments are shown together in Fig. 3.8 and the significantly greater (p<0.01) removal in the aurothiomalate treated animals is evident. As a consequence of the different level of excretion there was also a significant difference in the amount of retained radioactivity within the bodies at the end of the experiments with 30-35% of the thiomalic acid - C\(^{14}\) unaccounted for compared with only 2% remaining from the aurothiomalate - C\(^{14}\).

Chromatography and autoradiography of urine samples collected during the first 24 hours after injection showed that 2 major and at least 3 other compounds containing carbon-14 were excreted by the (1,4-\(^{14}\)C) thiomalic acid treated animals (Fig. 3.9). The densest spot on the autoradiograph had an Rf value which was identical to that of thiomalic acid. Urine samples collected from the sodium(1,4-\(^{14}\)C)aurothiomalate injected rats contained a greater number of radioactive compounds with 4 dense and at least three other spots (Fig. 3.9). Two of the dense areas of radioactivity coincided with the positions of sodium aurothiomalate and with thiomalic acid.

In subsequent experiments other small molecular weight thiol compounds were chromatographed together with the same urine samples in attempts to identify the radioactive components which were not established as either thiomalic acid or sodium aurothiomalate. The compounds investigated were cysteine and glutathione. Equimolar mixtures of these two with sodium aurothiomalate and with thiomalic acid were also prepared and chromatographed. Table 3.2 documents the Rf values obtained for these compounds and the mixtures and compares these values with those derived from the urine sample autoradiographs.

Both cysteine and glutathione travelled to give spots that were distinct from those of the two injected compounds. From the equimolar mixture of glutathione and thiomalic acid three spots were identified two of which corresponded to the individual components of the mixture while a third with an Rf of 39 suggested that a mixed species was also present. The mixture of cysteine and thiomalic acid however resolved into just two spots identified as the compounds added. From this observation it is inferred either that no association occurs between cysteine and thiomalic acid or that the chromatographic conditions cause the separation of any weak bonding between them. Likewise sodium
Fig. 3.8  Comparison of total carbon-14 excretion (Θ—) and excretion in the urine (Ο—) between rats given (1,4-14C)thiomalic acid and sodium(1,4-14C)aurothiomalate by intramuscular injection. Results expressed as mean ± standard deviation, n = 3, a = significant difference between the two groups (p<0.01).
Fig. 3.9  Radioactive metabolites in the urine of rats given (1,4-14C)thiomalic acid and sodium(1,4-14C)aurothiomalate by intramuscular injection. Metabolites separated by thin layer chromatography on silica gel G1500 using butanol, acetone, acetic acid, water (70:70:20:40) and located by autoradiography. Non dense spots shown by dotted outline.
Table 3.2 Rf values obtained by thin layer chromatography and autoradiography of rat urine samples collected for 24 hours after administration of (1,4-C)thiomalic acid and sodium(1,4-C)aurothiomalate, together with Rf values for thiol compounds and mixtures. Chromatography was on silica gel G1500 plates and developed with butanol, acetone, acetic acid, water (70:70:20:40:).

<table>
<thead>
<tr>
<th>Rf value</th>
<th>87</th>
<th>87</th>
</tr>
</thead>
<tbody>
<tr>
<td>84</td>
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<td>84</td>
</tr>
<tr>
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</tr>
<tr>
<td>77</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td></td>
<td></td>
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<tr>
<td>54</td>
<td>54</td>
<td></td>
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<tr>
<td>51</td>
<td>50</td>
<td></td>
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<tr>
<td>48</td>
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<td>47</td>
</tr>
<tr>
<td>44</td>
<td></td>
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</tr>
<tr>
<td>39</td>
<td>24</td>
<td></td>
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<tr>
<td>20</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
</table>

Samples:

1. thiomalic acid (tma)  
2. aurothiomalate (atm)  
3. glutathione (GSH)  
4. cysteine (cys)  
5. tma - GSH  
6. tma - cys  
7. atm - GSH  
8. atm - cys  
9. urine from tma-treated rats  
10. urine from atm-treated rats
aurothiomalate and glutathione did not appear to give a mixed species when the sample was chromatographed but with cysteine a third component to the chromatograph was generated when the gold compound was added.

Thus there is some evidence for complexes between thiomalic acid and glutathione and between cysteine and sodium aurothiomalate which are sufficiently stable to be chromatographed using the system employed here. Of the radioactive components separated in the urine samples only one could possibly be considered as coinciding with any of the mixed compounds. Urine from the rats given the radioactive gold compound had a dense spot at an Rf position of 76 which compared with the third cysteine-sodium aurothiomalate spot located with an Rf of 77.

The distribution of retained radioactivity within the tissues examined is shown in Fig. 3.10. The amount of radioactivity in each sample taken for analysis was determined by internal standardisation and calculated to express the result as nCi per gram of tissue. Since slightly different amounts of radioactivity were administered in the two experiments the results were then recalculated to give the percentage of the total administered dose per gram of tissue. These values were then multiplied by 1000 to avoid very small numbers. The recalculation therefore was \( \frac{nCi/g \times 100}{nCi \text{ injected} \times 1000} \). In this way direct comparisons can be made between the two groups of animals.

The radioactive distribution pattern was similar for both the carbon-14 labelled compounds. Surprisingly the highest concentration was found in the bone (a rib was taken for this analysis) with the second greatest level of activity present in the kidney. However, this was only one fifth the amount found in the bone. The liver, lungs, spleen and heart contained somewhat smaller concentrations with the lowest levels measured in fat, muscle, skin and the testis. In no tissue was radioactivity not detectable. Although the distribution was approximately the same in both sets of animals there were certain tissues - bone, skin and heart but particularly the kidney where retention of carbon-14 was significantly greater from the injected sodium(1,4-C)urothiomalate as compared with that found in the same tissues from the (1,4-C)thiomalic acid treated rats.
Radioactivity;  
\% of injected  
dose per gram  
of tissue \times 10^{-3} 

Fig. 3.10 
Tissue distribution of carbonyl  
72 hours after administration  
\((1,4^{14}C)\)tiomalic acid \(\Box\)  
or sodium\((1,4^{14}C)\)aurolithine  
\(\square\) to rats by intramuscular  
and skin injection. Results are expressed  
as mean and standard deviation.  
\(n = 3\). *Significant differences  
between groups.
The excretion of gold from the animals treated with sodium(1,4-\textsuperscript{14}C)aurothiomalate is shown in Fig. 3.11. During the first 24 hours, equal amounts of the metal were lost in the urine and faeces. Thereafter the urinary removal of gold fell off very rapidly whereas that found in the faeces was maintained at about the same level. At the end of the experiment i.e. 72 hours after the injection, about 30% of the administered gold had been recovered in the excreta with two-thirds of this in the faeces (Fig. 3.12).

Table 3.3 shows the concentrations of gold measured in the sera and in tissues removed after 72 hours. The concentrations were low and with the limited weight of material available gold was not detected in every sample. The greatest concentration was found in the kidney with a mean value of 1.24 µg/g. Smaller amounts were present in the liver, lung and spleen with even lower levels in the heart, skin, muscle and fat. The mean serum gold concentration was 10.7 µmol/l (210 µg/100 ml).

The total amount of gold present in each organ was calculated or, where organ weights were unavailable (e.g. for skin), from standard tables. (170) By adding the amount of gold measured in these tissues and the gold excreted, 45% of the injected gold was accounted for. The remaining 55% was presumably distributed throughout the organs not analysed.

Radioactivity measured at the site of intramuscular injection 24 hours after administration of the labelled compound was calculated as the percentage of the total dose per gram of muscle (NB - less than one gram of muscle was removed from the point of injection). From the rats injected with labelled sodium aurothiomalate, less than 2% of the dose was found per gram, whereas the samples from the animals given thiomalic acid contained considerably more, 11.3 ± 4.1% per gram.

2. Subcellular distribution of gold and carbon-14.

Enzymes

The activities of the marker enzymes in the subcellular fractions obtained by differential centrifugation are shown in Figs. 3.13 and 3.14. The distributions were similar in all experiments indicating that the administration of sodium aurothiomalate or thiomalic acid had no acute effect upon hepatocellular enzyme activity.
Fig. 3.11 Excretion of gold from rats for 72 hours after the administration of 4.2 μmoles sodium(1,4-14C)aurothiomalate by intramuscular injection. Histograms show mean ± standard deviation, n = 3.
Fig. 3.12

Cumulative excretion of gold from rats for 72 hours after the administration of 4.2 μmoles sodium (1,4-14C) aurothiomalate by intramuscular injection. Points show mean ± standard deviation, n = 3.
Table 3.3 Concentration of gold and carbon-14/gold ratios measured in serum and tissues of rats 72 hours after administration of sodium(1,4-\(^{14}\)C)aurothiomalate by intramuscular injection.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Gold (\mu g/g)</th>
<th>(^{14})C/Au ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.85 ± 0.11</td>
<td>14</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.24 ± 0.10</td>
<td>14</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.79 ± 0.09</td>
<td>11</td>
</tr>
<tr>
<td>Heart</td>
<td>0.59 ± 0.11</td>
<td>15</td>
</tr>
<tr>
<td>Lung</td>
<td>0.83 ± 0.16</td>
<td>9</td>
</tr>
<tr>
<td>Fat</td>
<td>0.16 ± 0.05</td>
<td>28</td>
</tr>
<tr>
<td>Skin</td>
<td>0.32 ± 0.05</td>
<td>21</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.18 ± 0.03</td>
<td>23</td>
</tr>
<tr>
<td>Serum ((\mu mol/\ell))</td>
<td>10.7 ± 1.32</td>
<td></td>
</tr>
<tr>
<td>Serum ((\mu g/100 \text{ ml}))</td>
<td>210 ± 26</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard deviation, \(n = 3\).

\(^{14}\)C/Au ratios were determined from the data shown in Fig. 3.10 for radioactivity and the gold concentrations given in this table.
Activity (cpm/mg) measured in subcellular fractions prepared from liver homogenates of rats given (1,4-¹⁴C) thiomalic acid, 2 (■) and 24 (■■) hours before killing (n = 5).

Carbon-14

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>M+L</th>
<th>Mic</th>
<th>Cyt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphatase</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5'-nucleotidase</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

N = nuclear, M + L = mitochondrial-lysosomal, Mic = microsomal, cyt = cytosol.
Fig. 3.14 Mean enzyme activities (U/ml/min), carbon-14 radioactivity (cpm/ml) and gold (µg/ml) measured in subcellular fractions prepared from liver homogenates of rats given sodium(1,4-14C)aurithiomalate, 2 (■) and 24 (□) hours before killing, n = 5. N = nuclear, M + L = mitochondrial-lysosomal, Mic = microsomal, cyt = cytosol.

- **Gold**
- **Carbon-14**
- **Succinic dehydrogenase**
- **Glucose-6-phosphatase**
- **5' nucleotidase**
- **Acid phosphatase**
No format for differential centrifugation produces exquisitely purified fractions of subcellular organelles (171) and some contamination between fractions was evident in these preparations.

The limited amounts of succinic dehydrogenase (SDH), acid phosphatase (AP) and glucose-6-phosphatase (G6P-ase) activities in the crude, nuclear fraction demonstrated the presence of both intact cellular material and the heavier cell fragments in addition to actual nuclear particles.

The SDH activities further indicated that, included with the nuclear and microsomal pellets, there were respectively a small number of heavier and lighter elements of the mitochondrial particles. However approximately 70% of the total SDH activity was present in the M + L fraction.

About 10% of the total AP activity was located with the microsomal fraction suggesting that lighter lysosomal particles separated with the homogenisation products of the endoplasmic reticulum. The limited proportion of G6P-ase activity (15% of the total) determined in the M + L fraction indicated that some of the microsomal material was trapped with the particulate matter derived from mitochondria and lysosomes.

The negligible activity of any of the enzymes assayed in the cytosol demonstrated that the high speed supernatant was free from contamination by organelles. This low level of enzyme activity was also apparent when expressed per milligram of protein and was not an artefact due to the large volume of the cytosol.

As a marker of membranous particles, 5'-nucleotidase (5-NT) activity was determined in all fractions although the M + L and especially the cytosol were distinct in having low levels (one tenth to one third) compared with the nuclear and microsomal fractions.

Despite this evident and anticipated cross-contamination between samples prepared by this classical procedure of differential centrifugation, each fraction appeared to be sufficiently pure (70-85% according to the major enzyme marker) to be able to demonstrate subcellular separation and distribution of the injected compounds.

Marker enzyme activities in the samples prepared from the sucrose gradient separation of the mitochondrial-lysosomal fraction also showed that there
was only trivial contamination from other elements (Fig. 3.15). The G6P-ase and 5-NT activities were of little consequence (\( \frac{1}{50} - \frac{1}{10} \)) compared with the levels found in other samples prepared by differential centrifugation. The lysosomes and mitochondria were well separated by the gradient as demonstrated by the position of acid phosphatase and SDH activity and also the distribution of protein. (172) The lighter lysosomes occupied fractions 1 to 6 and the mitochondria were located in fractions 11 to 18.

Radioactivity

Two hours after the injection of radioactive thiomalic acid 1.65% of the administered dose had been taken up by the liver. Of this radioactivity the major proportion, 76%, was located within the cytosol. The mitochondrial-lysosomal fraction contained about 15% while the residual 9% was distributed among the other organelles. One day later the radioactivity contained within the liver had decreased to 0.5% of the injected dose. There was an unequal loss from the various cellular sites and the cytosolic radioactivity then accounted for only 70% of the hepatic load while within the formed elements the relative proportions were somewhat increased (Table 3.4 and Fig. 3.13).

The subcellular distribution of carbon-14 in the animals given labelled sodium aurothiomalate was only slightly different to that of thiomalic acid with 80% present in the cytosol and about 10% in the mitochondrial-lysosomal elements (Fig. 3.14). As with the thiomalic acid the total content decreased by about two-thirds at 24 hours (Table 3.4).

Using the technique of sucrose density gradient centrifugation it was found that virtually all the radioactivity in the mitochondrial-lysosomal fraction was present in the mitochondria (Fig. 3.15).

Gold

Analysis of the cellular fractions of gold in the sodium aurothiomalate treated rats indicated that the metal initially accumulated in the cytosol but was cleared into organelles with the greatest concentration of gold per milligram of protein found in the mitochondrial-lysosomal material. The clearance was particularly efficient so that 24 hours after the injection, approximately 10% of the cellular gold was contained within the cytosol (Table 3.4). From the sucrose density gradient separation of the
Fig. 3.15 Mean enzyme activities (U/ml/min), carbon-14 radioactivity (cpm/ml) and gold (μg/ml) measured in rat liver mitochondrial-lysosomal fractions separated by sucrose density gradient centrifugation 2 hours after administration of sodium(1,4-L14C) aurothiomalate, n = 3. (Some representative standard deviation values are also shown).
Table 3.4 Retention of gold and carbon-14 in the liver and liver subcellular fractions in rats given (1,4-$^{14}$C)thiomalic acid and sodium(1,4-$^{14}$C)aurothiomalate.

<table>
<thead>
<tr>
<th></th>
<th>Thiomalic acid treated rats</th>
<th>Sodium aurothiomalate treated rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hrs</td>
<td>24 hrs</td>
</tr>
<tr>
<td><strong>CARBON-14</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of injected dose found in liver</td>
<td>1.65 ± 0.5</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>% of liver $^{14}$C found in each fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>76.0 ± 8.2</td>
<td>70.0 ± 5.9</td>
</tr>
<tr>
<td>Mitochondria-lysosomes</td>
<td>14.9 ± 1.7</td>
<td>17.0 ± 1.2</td>
</tr>
<tr>
<td>Other fractions</td>
<td>9.1 ± 1.1</td>
<td>13.0 ± 1.3</td>
</tr>
<tr>
<td><strong>GOLD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of injected dose found in liver</td>
<td>0.8 ± 0.2</td>
<td>3.6 ± 1.0</td>
</tr>
<tr>
<td>% of liver Au found in each fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>56.8 ± 6.3</td>
<td>10.3 ± 1.6</td>
</tr>
<tr>
<td>Mitochondria-lysosomes</td>
<td>15.8 ± 3.5</td>
<td>44.8 ± 4.2</td>
</tr>
<tr>
<td>Other fractions</td>
<td>27.4 ± 3.3</td>
<td>44.9 ± 4.0</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard deviation, n = 5.
mitochondrial-lysosomal fraction the gold was found in the tubes containing acid phosphatase activity, i.e. within the lysosomes (Fig. 3.15).


Secretion of components into the bile may be expressed as weight per minute per unit of body weight or weight per unit volume of bile. Provided that bile flow is constant throughout the course of an experiment either technique is valid. In the experiments conducted here, biliary flow, measured by the weight of bile collected, was found to remain constant for at least four hours.

The clearance of radioactive carbon-14 from the plasma of rats given (1,4-\textsuperscript{14}C)thiomalic acid was extremely rapid (Figs. 3.16 and 3.17). Within three minutes of intravenous injection, only about 10% of the administered radioactivity was present within the blood (assuming a blood volume of 12 ml in a 200 g rat and rapid equilibration of the thiomalic acid between plasma and cells). That the low plasma levels of carbon-14 were due to efficient clearance and not faulty injection is suggested by - (i) blood was drawn into the syringe before the injection was given, (ii) in one experiment the radioactivity retained in the liver at the conclusion was measured and found to represent approximately 10% of the injected activity. Therefore a reasonable proportion of the dose had been transported from the neck to the abdomen - i.e. through the blood. The half life calculated from the disappearance curve (Fig. 3.17) was 14 minutes. Very low levels of radioactivity were measured in the bile (Fig. 3.16) and none was detected later than 120 minutes after injection. When the (1,4-\textsuperscript{14}C)thiomalic acid was preadministered 24 hours before bile was collected, no radioactivity could be measured in either the plasma or the bile.

With the radioactive gold compound however the plasma carbon-14 half life was somewhat longer (Figs. 3.17 and 3.18). At 3 minutes post-injection more than 40% of the administered material was still within the circulation. The disappearance curve showed an initial distribution phase lasting approximately 60 minutes followed by a steady loss of activity with a \(t_\frac{1}{2}\) of 70 minutes (Fig. 3.17) and radioactivity could be detected in the plasma for at least four and a half hours.

Although there was more radioactivity in the plasma compartment a similar proportion of the administered carbon-14 was secreted into the bile as was found with the labelled thiomalic acid (Fig. 3.16).
Fig. 3.16  Carbon-14 measured in plasma and bile of 2 rats given (1,4-14C)thiolic acid by intravenous injection. Animal 1; plasma...+, bile ——. Animal 2; plasma...•••, bile ——.
Fig. 3.17 Plasma clearance of carbon-14 from rats given \((1,4^{14}\text{C})\) thiomalic acid or sodium\((1,4^{14}\text{C})\) aurothiomalate by intravenous injection.
Fig. 3.18  Carbon-14 measured in plasma and bile of rats given sodium(1,4-14C)aurothiomalate by intravenous injection. Results show mean ± standard deviation, (n = 3). Note - different scales for plasma and bile and in Fig. 3.16.
The disappearance of gold from the blood and its appearance in bile are shown in Fig. 3.19 and a picture completely different to that seen for carbon-14 was found. Approximately 40% of the administered gold was present in the plasma at the time when the first blood sample was collected (3-5 minutes after injection). This calculation assumes a blood volume of 12 ml of which the plasma, where gold is confined, is about 6 ml. After 30-40 minutes the plasma gold concentration was increased by about 10% but thereafter decreased gradually. The plasma gold half life calculated from this linear section of the curve was 242 minutes. Biliary gold concentrations were approximately 1% of the plasma concentration. A peak of secretion occurred 10-15 minutes later than the point at which maximum plasma gold levels were attained. There was then a gradual decrease in the biliary gold output which paralleled the fall in plasma gold although at three hours post-injection a slight increase in biliary gold was evident. The total gold secreted during the first three hours following intravenous injection was 0.55 ± 0.16 μg or 0.44% of the injected gold.

Thin layer chromatography and autoradiography of bile collected during the first 20 minutes following injection of sodium(1,4-14C)auromethiomalate revealed just a single radioactive component with an Rf value of 84 which was the same as was found for thiomalic acid (Fig. 3.20).


Elution of protein: The purpose of the experiment was essentially to separate material bound to albumin (approximate molecular weight 70,000) from compounds with very low molecular weights—less than 1,000. Therefore a very simple separation system was all that was required. Using G75 the albumin eluted as a sharp peak between fractions 10 and 16 (collection of the first fraction was commenced when all the sample had just entered the column). The elution volume Ve was 25 ml and was well away from the bed volume of the column at which point the low molecular weight, unbound compounds appeared (Fig. 3.21). Thus this separation system was satisfactory.

The concentrations of gold and albumin in the time course experiment were selected to give approximately the values which would be found in the plasma of a patient soon after receiving an intramuscular injection of sodium aurothiomalate. The distribution of gold and carbon-14 between the albumin and non-albumin bound fractions following Sephadex separation is shown in Table 3.5. The major proportion of the gold but no more than 10% of the radioactivity was associated with the albumin at 30 minutes after addition.
Fig. 3.19 Gold measured in plasma and bile of rats given sodium aurothiomalate (2.5 mg/kg) by intravenous injection. 
Results show mean ± standard deviation, (n = 3).
Fig. 3.20 Autoradiograph of thin-layer chromatographic separation of bile collected from rat for 20 minutes after intravenous injection of sodium(1,4-\textsuperscript{14}C)aurothiomalate. Chromatographic conditions as given in Table 3.2.

- **a** = position of sodium aurothiomalate (May & Baker Ltd.)
- **b** = position of thiomalic acid (Sigma Chemical Co.)
- **c** = position of biliary radiolabelled compound on autoradiograph
Fig. 3.21 $OD_{280}$nm, gold and carbon-14 in eluate from Sephadex G75 gel chromatography column (20 x 250 mm). Albumin (4% w/v) and sodium aurothiomalate (36.6 µmol/l) + tracer sodium(1, 4-¹⁴C)aurothiomalate incubated for 30 minutes at room temperature and 1.5 ml applied to column. Eluted with phosphate buffered saline at 4.0 ml per hour. Each fraction collected for 30 minutes.
Table 3.5 Gold and carbon-14 distribution between albumin-bound and non-bound fractions following incubation for increasing periods of time of albumin (40 g/l) and sodium aurothiomalate (35.5 μmol/l with 14C-labelled tracer aurothiomalate). Separation of bound and non-bound fractions achieved by gel filtration on Sephadex G75.

<table>
<thead>
<tr>
<th>Incubation period</th>
<th>Recovery % 14C</th>
<th>Au</th>
<th>Protein Bound % 14C</th>
<th>Au</th>
<th>Non-protein Bound % 14C</th>
<th>Au</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>95.5 ± 3.0</td>
<td>97.2 ± 3.1</td>
<td>7.4 ± 2.3</td>
<td>73.6 ± 2.3</td>
<td>92.6 ± 2.4</td>
<td>26.4 ± 2.3</td>
</tr>
<tr>
<td>5 mins</td>
<td>97.2 ± 2.1</td>
<td>102.5 ± 2.8</td>
<td>5.5 ± 1.4</td>
<td>73.0 ± 1.9</td>
<td>94.3 ± 1.1</td>
<td>27.0 ± 1.9</td>
</tr>
<tr>
<td>15 mins</td>
<td>103.1 ± 2.8</td>
<td>96.7 ± 3.3</td>
<td>8.5 ± 3.0</td>
<td>75.9 ± 2.0</td>
<td>91.5 ± 3.0</td>
<td>24.1 ± 2.0</td>
</tr>
<tr>
<td>30 mins</td>
<td>103.9 ± 2.7</td>
<td>98.6 ± 2.2</td>
<td>8.3 ± 2.4</td>
<td>76.7 ± 2.2</td>
<td>91.7 ± 2.4</td>
<td>23.3 ± 2.2</td>
</tr>
<tr>
<td>24 hrs</td>
<td>97.8 ± 2.5</td>
<td>100.8 ± 3.0</td>
<td>21.1 ± 4.1</td>
<td>92.5 ± 1.1</td>
<td>78.9 ± 4.1</td>
<td>7.5 ± 1.0</td>
</tr>
<tr>
<td>7 days</td>
<td>96.7 ± 2.2</td>
<td>103.1 ± 2.5</td>
<td>13.0 ± 2.7</td>
<td>89.1 ± 2.6</td>
<td>87.0 ± 1.6</td>
<td>10.9 ± 2.6</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard deviation, n = 3.
of the drug. Distribution at earlier times was very similar but there was relocation with longer periods of incubation. The proportion of albumin bound gold increased to approximately 90% while a greater proportion of the carbon-14 moved onto the albumin only to be displaced after 7 days.

Fig. 3.22 shows that divorce of gold from carbon-14 was not effected merely by passage of the sodium aurothiomalate through the Sephadex. The elution profile of these two components of the labelled compound were identical when albumin was omitted from the medium and showed no evidence of separation.

Thiomalic acid does not appear to bind to albumin. Fig. 3.23 shows that the radioactivity was well separated from the protein and appeared at the solvent volume, Vs. The inference from these data is that in the sodium aurothiomalate experiments any carbon-14 which was found in association with protein can only be as the intact aurothiomalate and not as thiomalic acid.

In Table 3.6 the distribution of gold and carbon-14 at different albumin : sodium aurothiomalate molar ratios is shown. Where the proportion of protein to drug was high, all the detectable gold was protein bound but with increasing concentration of sodium aurothiomalate, progressively more of the total appeared in the unbound fraction. A similar shift in the distribution of radioactivity also occurred although far less was initially associated with the protein. Using the data obtained the amounts of gold and carbon-14 bound to albumin were plotted according to the method of Scatchard to determine the binding characteristics (Figs. 3.24 and 3.25). These plots are characteristic of multi-component systems and show the typical features of high affinity - low capacity and low affinity - high capacity binding sites. Apparent affinity constants and binding capacities were calculated according to the equations derived by Scatchard and are shown in Table 3.7.

Affinity at the main binding site was greater for gold, $1.02 \times 10^3$ compared with carbon-14 $0.42 \times 10^3$ and similarly the maximum binding capacity for gold was greater than for carbon-14 (4.7 and 5.5 μmol/mmol respectively). Binding to the secondary site(s) exhibited similar differential results for both the gold and carbon-14.
Fig. 3.22 Gold and carbon-14 in eluate from Sephadex G75 gel chromatography column (20 x 250 mm). Sodium aurothiomalate (36.6 μmol/l) + tracer sodium(1,4-14C) aurothiomalate added to column. Eluted with phosphate buffered saline at 4.0 ml per hour. Each fraction collected for 30 minutes.
*Fig. 3.23* $\text{OD}_{280\text{nm}}$ and carbon-14 in eluate from Sephardex G75 gel chromatography column (20 x 250 mm). Albumin (4% w/v) and thiomalic acid (36.0 μmol/l) + tracer (1,4-$^{14}$C)thiomialic acid incubated for 30 minutes at room temperature and 1.5 ml applied to column. Eluted with phosphate buffered saline at 4.0 ml per hour. Each fraction collected for 30 minutes.
Table 3.6 Gold and carbon-14 distribution between albumin-bound and non-bound fractions following 30 minutes incubation of albumin (40 g/%) with increasing concentrations of sodium aurothiomalate (with 14C-labelled tracer aurothiomalate). Separation of bound and non-bound fractions achieved by gel filtration on Sephadex G75.

<table>
<thead>
<tr>
<th>Gold concn µmol/</th>
<th>Albumin/atm ratio</th>
<th>Recovery % C14</th>
<th>Recovery % Au</th>
<th>Protein Bound % C14</th>
<th>Protein Bound % Au</th>
<th>Non-protein Bound % C14</th>
<th>Non-protein Bound % Au</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.66</td>
<td>161 : 1</td>
<td>95.1 ± 3.8</td>
<td>106.6 ± 8.8</td>
<td>19.2 ± 3.2</td>
<td>100.0</td>
<td>80.5 ± 3.7</td>
<td>0</td>
</tr>
<tr>
<td>18.30</td>
<td>32 : 1</td>
<td>97.8 ± 4.1</td>
<td>98.5 ± 4.7</td>
<td>13.5 ± 1.1</td>
<td>95.2 ± 2.0</td>
<td>86.5 ± 1.1</td>
<td>4.8 ± 2.0</td>
</tr>
<tr>
<td>36.60</td>
<td>16 : 1</td>
<td>103.9 ± 2.7</td>
<td>98.6 ± 2.2</td>
<td>8.3 ± 2.4</td>
<td>76.7 ± 2.2</td>
<td>91.7 ± 2.4</td>
<td>23.3 ± 2.2</td>
</tr>
<tr>
<td>73.20</td>
<td>8 : 1</td>
<td>95.9 ± 3.2</td>
<td>97.3 ± 5.3</td>
<td>5.7 ± 1.6</td>
<td>58.4 ± 1.9</td>
<td>93.6 ± 0.7</td>
<td>41.6 ± 1.9</td>
</tr>
<tr>
<td>182.9</td>
<td>3.2 : 1</td>
<td>88.8 ± 3.7</td>
<td>95.9 ± 4.6</td>
<td>4.8 ± 0.9</td>
<td>34.4 ± 0.6</td>
<td>95.2 ± 0.9</td>
<td>64.3 ± 1.7</td>
</tr>
<tr>
<td>365.9</td>
<td>1.6 : 1</td>
<td>93.6 ± 5.2</td>
<td>91.5 ± 4.1</td>
<td>4.2 ± 1.8</td>
<td>19.2 ± 0.7</td>
<td>95.8 ± 1.8</td>
<td>80.8 ± 0.7</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard deviation, n = 3.
Scatchard plot of gold binding sites on albumin. The ratio of the bound to free gold is on the vertical axis. The association constant of the high affinity site was obtained from the slope of the straight line at low gold concentrations after correction for the contribution of the low affinity site(s) at higher gold concentrations. The maximum binding capacities were calculated from the intercepts on the horizontal axis.
Carbon-14 (aurothiomalate) bound; μmol/mmol albumin

Fig. 3.25
Scatchard plot of carbon-14 (aurothiomalate) binding sites on albumin. The ratio of the bound to free carbon-14 is on the vertical axis. The association constant of the high affinity site was obtained from the slope of the straight line at low aurothiomalate concentrations after correction for the contribution of the low affinity site(s) at higher aurothiomalate concentrations. The maximum binding capacities were calculated from the intercepts on the horizontal axis.
Table 3.7 Apparent affinity constants and binding capacities of the gold and carbon-14 binding sites on human albumin. Site I is a high affinity, low capacity site. Site II is one (or representative of several) low affinity, high capacity site. The contributions of the low affinity binding site(s) were subtracted from the results of the high affinity binding sites before completing the calculations. Results were calculated from Figs. 3.24 and 3.25.

<table>
<thead>
<tr>
<th></th>
<th>Maximum binding capacity (μmol/mmol albumin)</th>
<th>Apparent affinity constant (L/M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Site I</td>
<td>Site II</td>
</tr>
<tr>
<td>Gold</td>
<td>47</td>
<td>140</td>
</tr>
<tr>
<td>Carbon-14</td>
<td>5.5</td>
<td>61</td>
</tr>
</tbody>
</table>
3.3.4 Discussion

1. Disposition and excretion of gold and carbon-14.

If a rapid separation of gold from the carrier takes place as has hitherto been assumed, the excretion and tissue distribution of radioactive carbon would be the same in rats injected with either sodium aurothiomalate or thiomalic acid. The results obtained are inconsistent with this assumption.

(i) during the initial 24 hours, which is the period when metabolic activity was particularly dynamic, more aurothiomalate-carbon-14 than thiomalate acid-carbon-14 was removed by the kidney (Fig. 3.8) and there was an increased deposition of radioactivity in the kidney, bone and skin (Fig. 3.10).

(ii) the balance of radioactivity unaccounted for by excretion and tissue retention was approximately 35% in the thiomalate treated rats and only 1-2% in the gold treated animals.

(iii) the labelled compounds detected in the urine by autoradiography (Fig. 3.9) were dissimilar and there were fewer in the samples from the non-gold treated rats.

However, the considerable differences in the patterns of excretion of gold and radioactive carbon in the rats injected with sodium\(1,4-^{14}\text{C}\)aurothiomalate indicates that much of the gold is removed from the labelled portion of the drug (thiomalate) within a few hours.

An explanation for the contrasting rates of excretion of radioactive carbon between the two groups of animals and also for the apparent enigma concerning the carbon-14 which was unaccounted for in the rats given labelled thiomalic acid may be derived from the results of the measurements of radioactivity retained in the muscle at the site of injection.

The free sulphydryl group on thiomalic acid would appear to allow the formation of stable complexes within muscle. When this group is masked by the gold atom the molecule is less likely to be retained and entry to the circulation is virtually complete. Therefore the lower levels of radioactivity measured in the excreta and tissues of rats given thiomalic acid could be merely a reflection of the \textit{de facto} reduced dose.
This proposal however does not provide an explanation for the patterns of urinary metabolites which were found and a rapid removal of all the gold from the thiomalate upon entry into the circulation cannot therefore be assumed.

2. Subcellular distribution of gold and carbon-14.

The very similar patterns of intracellular distribution of carbon-14 (Figs. 3.13 and 3.14) when administered either as sodium aurothiomalate or as thiomalic acid suggests two possibilities. Gold may be stripped from the thiomalate upon entry into the body so that essentially the same labelled compound is metabolised in both experiments. Alternatively, while some or all of the aurothiomalate remains intact within the circulation and enters the liver, the gold is removed from thiomalate during or very soon after passage across the cell membrane so that the liver cell is exposed only to labelled thiomalic acid.

Very little (less than 25%) of the radioactive carbon within the liver became incorporated into the formed structures of the cell and most of this was located in the mitochondria (Fig. 3.13, Table 3.4). The mitochondria of Kupffer cells are much smaller than mitochondria of hepatocytes. Therefore if the thiomalate-carbon-14 was primarily in Kupffer cells the peak of radioactivity in Fig. 3.15 would be biased towards the lower side of the peak of SDH activity. However, the exact parallelism observed between carbon-14 and enzyme activity indicates that the labelled material is within all cells or even predominantly within the more numerous hepatocytes.

Incorporation of gold into the liver (Table 3.4) was similar to that found by other workers (95,153), and is consistent with the results in Table 3.3. Histochemical and electromicrographic examinations of the liver tended to show most gold within the hepatocytes. (156) Furthermore, since biliary excretion occurs and is presumably responsible for at least some of the faecal elimination of gold (Chapter 1 and Figs. 3.11 and 3.12), metabolism of gold must be mainly within the hepatocyte.

However, removal of gold from the circulation has been attributed to the activity of the hepatic reticulo-endothelial cells and the concentration of gold within the Kupffer cells was reported to be double (per unit of protein) that found in hepatocytes. (173) Even so, the majority of the total hepatic gold was located within the more numerous fundamental liver cell
Some hepatocellular gold may nonetheless be passed from Kupffer cells as is known to occur with iron.

Therefore, while the phagocytic activity of the Kupffer cells may result in some gold and carbon-14 uptake, quantitatively the incorporation into hepatocellular tissue, either directly or via the Kupffer cells will be of greater importance in the metabolism of aurothiomalate.

The results presented in Table 3.4 and Fig. 3.14 and Fig. 3.15 are consistent with the findings of Lawson et al. (153) that within the liver cell there is a concentration of gold into the lysosomes within a few hours of intra-peritoneal injection of sodium aurothiomalate. From Table 3.4 it is seen that in the early phase of gold metabolism, cytosolic gold represents a high proportion of the hepatic gold content. Lawson et al. (153) further examined this fraction and found the gold associated with four separate complexes one of which was only detected when the gold was pre-administered by at least 7 days.

This fraction was not further characterised and the authors avoided speculation as to whether it might represent a thionein protein although its appearance only after preliminary exposure to gold, the low absorbance at 280 nm and the approximate molecular weight of 10,000 are consistent features of thionein. Evidence for the formation of gold-thionein in the kidney following exposure of rats to sodium aurothiomalate is presented in the next chapter.

From the data of Lawson et al. (153) and that given in Figs. 3.13 - 3.15 and Table 3.4 the following course of events can be suggested for the sodium aurothiomalate entering the liver. As aurothiomalate crosses the cell membrane into the cytosol the cellular proteins-albumin and ligandin, strip gold from thiomalate. Where previous gold exposure has occurred a significant proportion of the gold also binds to a low molecular weight protein that may be metallothionein. Some metal also complexes with thiol compounds such as glutathione and cysteine. Gold which is removed from thiomalate within the circulation may be expected to follow a similar course upon entry into the hepatocyte.

From the cytosol the major fraction of hepatocellular gold is eventually concentrated in lysosomes so that within twenty four hours of injection most of the hepatic gold is found within these subcellular structures (Table 3.4).
Thiomalate, either liberated intracellularly from gold or taken in from the plasma largely remains within the cytosol. Approximately 10% enters the mitochondria where some conversion to carbon dioxide takes place (see Figs. 3.6 and 3.7) but most is static within the cell (Table 3.4). Intracellular location of thiomalic acid is consistent with the observations on the binding of the compound at the site of injection and the very short plasma half life following intravenous injection of \((1,4^{14}\text{C})\) thiomalic acid.


The most remarkable feature of the experiments with thiomalic acid was the very rapid disappearance from blood (Fig. 3.16). Removal from the circulation at this rate could be achieved by intracellular sequestration by compounds with extremely high avidity for thiomalic acid. Alternatively, dilution may take place by equilibration throughout the total body water as occurs with e.g. fucose, \(p\)-amino hippuric acid. In either case entry of the compound into the bile would be limited, as was found with thiomalic acid (Fig. 3.16) and would be accomplished by diffusion and leakage across the hepato-biliary membrane.

Movement of carbon-14 was quite different when the thiol group was masked by gold. The results obtained (Fig. 3.18) are in agreement with those presented above (Figs. 3.8 - 3.10) which provided evidence for the maintenance of some intact drug and do not support the hypothesis that gold is immediately lost from the thiomalate moiety upon entry into the circulation. Disappearance of radioactivity was retarded compared with the non-gold compound, possibly due to the formation of a mixed albumin-gold-thiomalate species (see below). By contrast however, as soon as radioactivity derived from aurothiomalate has entered the liver, it follows a course which is very similar to that of thiomalic acid-carbon-14 with a slow trickle into the bile. This similarity in the handling of carbon-14 from both thiomalic acid and aurothiomalate was also found in the subcellular distribution experiments and suggests that the gold portion of any intact drug which is taken into the hepatocyte is rapidly removed to expose the thiol group.

Examination of the biliary components by thin-layer chromatography and autoradiography provided further evidence to support this suggestion with a failure to detect the presence of sodium aurothiomalate carbon-14.
even though a radioactive compound with the same Rf as thiomalic acid was found (Fig. 3.20).

The plasma gold half life calculated from the data available during the three hours after intravenous injection was 242 minutes. This figure is compared with the half times determined by other workers in Table 3.8 from which it is apparent that the movement of gold from blood follows a complex course. Fig. 3.19 shows that within the first hour there are at least two different phases with removal of gold from the plasma (60% of the injected dose) during the first few minutes followed by a return back into the blood so that there is a rebound in plasma concentration before a third, stable, phase is established which exists for at least a further two hours.

This early period of gold pharmacokinetics has not been examined in any detail by other workers who have generally confined their attention to the disappearance of gold over much longer periods, usually commencing several hours after the injection. Such studies have produced data which effectively integrate any short-term subtle changes which occur during these times. Harth (83) recognised that there were at least two components to the long-term decline in plasma gold. He differentiated a period up to 36 hours which was characterised by a much shorter half life than during the subsequent 5-6 days (Table 3.8).

A sequence of events to describe the movements of gold in plasma observed during these short-term experiments can be envisaged. The injected sodium aurothiomalate distributes throughout the plasma and extracellular fluid but binding to albumin draws some gold back into the plasma compartment. These fluctuations, which last for about one hour, are superimposed upon the removal of gold into tissues such as the kidney and liver. From the data obtained during the long-term experiments (77,83,84) there appears to be a certain amount of re-release of gold from the tissue sites back into the circulation several days after the injection of aurothiomalate and therefore the measured half life becomes longer as observations over more extended periods are made.

Gold which enters the tissues during the immediate post-injection period passes passively across the biliary-hepatocyte membrane so that the concentration of gold in bile reflects, with a short time-lag, the fluctuations in plasma gold concentration. It is seen in Fig. 3.19 however
Table 3.8 Calculated half lives for plasma gold clearance following administration of sodium aurothiomalate to rats (intravenous and intraperitoneal injections) and to man (intramuscular injections).

<table>
<thead>
<tr>
<th>Period of observation following injection (hrs)</th>
<th>Route of administration</th>
<th>Half life (hrs)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 - 3</td>
<td>i.v.</td>
<td>4</td>
<td>This work</td>
</tr>
<tr>
<td>4 - 36</td>
<td>i.m.</td>
<td>9.7</td>
<td>Harth (83)</td>
</tr>
<tr>
<td>1 - 168</td>
<td>i.p.</td>
<td>36</td>
<td>Lawson et al. (153)</td>
</tr>
<tr>
<td>36 - 168</td>
<td>i.m.</td>
<td>143</td>
<td>Harth (83)</td>
</tr>
<tr>
<td>24 - 192</td>
<td>i.m.</td>
<td>108</td>
<td>Gottlieb et al. (77)</td>
</tr>
<tr>
<td>24 - 240</td>
<td>i.m.</td>
<td>132</td>
<td>Gerber et al. (84)</td>
</tr>
</tbody>
</table>

i.v. = intravenous, i.p. = intraperitoneal, i.m. = intramuscular
that the biliary gold concentration appears to be increasing at three hours even though the plasma gold concentration is falling. This increase could be due to the start of a breakdown in hepato-biliary function in the rats maintained in the experimental, anaesthetised condition or it could be the first sign of the appearance in bile of gold previously accumulated in lysosomes.

Enrichment of the biliary concentration by a mechanism such as discharge of accumulated lysosomal gold would account for the disparity between the amount of gold found in the faeces (10%) in the metabolic experiments described above (Fig. 3.12) and the calculated rate of secretion of gold into the bile. If gold continued to be secreted at the same rate as was measured in the first three hours, and if there were no entero-hepatic circulation, the total excretion after 24 hours would be approximately 3.5% of the administered dose.


The results presented indicate that even in a relatively simple in vitro system, set up at physiological pH, some 'metabolism' of sodium aurothiomalate is effected by albumin. Given therefore that there is considerable separation of gold from the thiomalate when the two compounds are incubated, one can anticipate three possible protein bound species being formed i.e. albumin-Au-tma, albumin-Au and albumin-tma. The results from the experiment with albumin and thiomallic acid showed however that any association between these two compounds was very labile and complete separation is readily achieved by passage through Sephadex. Therefore it can be concluded that in the binding of carbon-14 to albumin which was demonstrated with sodium aurothiomalate, the gold atom must form an integral part of the complex i.e. albumin-Au-tma. Since more gold than carbon-14 was bound to albumin the metal-protein complex, albumin-Au must also exist.

The nature of the non-protein bound species can also be considered. From our knowledge of the chemistry of gold the existence of 'free Au(1)' can be discounted and any gold in this fraction must therefore exist with a suitable ligand, i.e. in this simple system as unchanged sodium aurothiomalate. Unlike gold the majority of radioactivity remained within the non-protein bound compartment, therefore most of the carbon-14 would represent the drug after removal of the gold i.e. thiomalic acid.
It is therefore concluded that when sodium aurothiomalate is added, *in vitro*, to albumin, four compounds are formed. The relative proportion of each are shown in Fig. 3.26. Although this static system has only a limited resemblance to the complex dynamic environment which is the plasma of a patient with rheumatoid arthritis it does nonetheless allow for discussion and arguments to be developed and to include compounds which are known to form. The results presented indicate that about two-thirds of the sodium aurothiomalate is very rapidly disrupted with the gold sequestered by albumin. Some drug however remains intact and is divided between the bound and unbound fractions. A small amount of relocation and further disruption of the residual sodium aurothiomalate occurred but was very slow and presumably therefore has little significance to the situation that pertains *in vivo*.

Rapid formation of albumin-gold complexes were similarly demonstrated by Gerber (8) and McKinley (3). McKinley (3) also showed that the pH of the reaction environment was important and that in an unbuffered system at pH 5.1, association (monitored by circular dichroism spectra) was initially very weak and several days incubation were required before equivalent size peaks were observed.

When Gerber (8) and Danpure (161) incubated albumin and sodium aurothiomalate they were unable to detect release of thiomalic acid from the complex, (measurements were made *in situ*, not following a separation of bound and unbound components). This would suggest that either (a) the sodium aurothiomalate binds to albumin without being metabolised or that (b) an albumin-Au species is formed but the thiomalate is retained within the environment of the complex by weak bonding forces. The interaction between aurothiomalate and cysteine was investigated by Danpure (161) as a model for the interaction at the albumin binding site. Under these conditions thiomalic acid was released from the cysteine-gold complex suggesting that option (b) is the most appropriate explanation for the observations of Gerber (8) and Danpure (161). This option, a weak attraction of thiomalate to albumin, is not inconsistent with the results presented in Fig. 3.23. Passage through the Sephadex during gel chromatography might provide appropriate conditions for breakdown of the weak bonding forces with the clear separation of albumin and thiomalic acid.
Fig. 3.26 Representation of "metabolism" of sodium aurothiomalate by albumin. Data taken from Table 3.5. Number within symbol indicates % of material (gold or $^{14}\text{C}$) present in each fraction.

In vitro Incubation time

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Albumin bound</th>
<th>Non-bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 mins</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>24 hrs</td>
<td>76 22</td>
<td>71-5</td>
</tr>
<tr>
<td>7 days</td>
<td>11 11</td>
<td>76</td>
</tr>
</tbody>
</table>

= albumin
= aurothiomalate
= gold
= thiomalate
Thiomalic acid, bound to albumin either through gold or weakly held close to the complex would remain available for the formation of further mixed thiol species or for participation in the thiol exchange reactions which occur in solution and could be expected to occur in vivo (Chapter 1).

Characterisation of the albumin-drug binding sites (Table 3.7) produced results which are similar to those determined by other workers (162,163) even though different techniques were employed to differentiate the bound and unbound species. The detail of the multi component nature of gold binding to albumin has not been extensively explored although it is evident from the Scatchard and similar plots prepared by these authors and from Figs. 3.24 and 3.25 that there are two or more low affinity sites.

With the exception of the attempts to measure release of thiomalic acid (which were referred to above), binding of the non-gold component of sodium aurothiomalate to albumin has not previously been investigated. The maximum binding capacities observed at sites I and II for both gold and radioactivity are consistent with the hypothesis that binding of this non-gold component is through the metal atom and not directly onto the albumin molecule. The key role of gold in the binding of aurothiomalate at site I is emphasised by the greater avidity demonstrated for the metal in comparison with the thiomalate label which is also consistent with the formation of an albumin-Au-thiomalate complex.

Assembling together the results from the metabolic, subcellular distribution, biliary excretion and in vitro binding experiments, it becomes possible to propose an overview which describes many features of the metabolism of sodium aurothiomalate (Fig. 3.27) and to present some ideas concerning the effect of thiomalate upon reactions involving gold. Much of the information used has not previously been available and certain features are therefore novel with no support from independent data. Since particular attention has been focussed upon the liver this description is necessarily directed at events in this tissue and reference to other sites - particularly the joints can be by implication and extrapolation only and it is important to differentiate between generalisations and conclusions from specific experiments.
Fig. 3.27 Representation of features in the cellular metabolism of sodium aurothiomalate.

PLASMA

albumin

Au atm

atm
tma

TCA cycle → CO₂

bile canaliculus

soluble cytosolic complexes e.g. with cysteine glutathione etc.

Au

albumin

ligandin

thionein

lysosomes

Au

14C

Au

Au

tma = thiomalic acid
atm = aurothiomalate
Intramuscular injection provides an efficient means for the administration of aurothiomalate. The almost full recovery of radioactivity (Fig. 3.8) indicated that very little residue was left at the application site, an inference which was confirmed by examination of muscle at the point of injection. Most or all of the aurothiomalate is absorbed from the injection site unchanged. The carbon-14 recovery and retention experiments with thiomalic acid suggest that if gold were removed from thiomalate intramuscularly a large proportion of the labelled material would not have become available for circulation, tissue uptake and excretion. Because the aurothiomalate enters the blood unchanged the administration of the compound by intravenous injection can be claimed to be valid with the qualification that rate of entry is much more rapid than occurs from an intramuscular depot.

A major redistribution of gold occurs within a few minutes of entry into the blood and if the results from the *in vitro* system also apply *in vivo* approximately two thirds of the gold is removed from the thiomalate and is complexed with albumin (Table 3.5). The unchanged aurothiomalate distributes between albumin and other complexes (presumably cysteine and other thiol-containing compounds). The non-protein bound fraction is greatest with the higher total gold concentrations (Table 3.6). These high concentrations are found soon after injection which is also the period when greatest renal excretion occurs. It is probable therefore that gold transported as the non-protein bound fraction in the plasma is filtered at the glomerulus and only some (if any) is reabsorbed by renal tubular cells. The importance of other possible mechanisms e.g. incorporation of gold into renal protein and subsequent excretion, dissociation of albumin-gold complex in the kidney, excretion of gold complexes formed in other organs and later brought to the kidney, cannot be judged but may become more relevant at much later times.

The liberated thiomalate has a very short existence in plasma (Figs. 3.16 and 3.17) and almost immediately is dispersed throughout the total body water, entering all tissues. Some gold (it is not apparent which plasma fraction(s)) also became incorporated intracellularly. Within the liver it appears to be irrelevant whether gold enters with the thiomalate ligand or as some other complex since the hepatic metabolism of carbon-14 from either thiomalate or aurothiomalate is identical and it is concluded therefore that intracellularly the thiomalate is immediately separated from the gold.
Intracellular thiomalate therefore is derived from two possible sources, that released as gold forms a complex with plasma albumin and that which is given up actually within the cell. Most of the thiomalate remains in the cytosol with about 10% entering mitochondria where further metabolism takes place with the eventual pulmonary excretion of carbon dioxide. Cytosolic thiomalate is removed to the kidney and is excreted (Figs. 3.6 and 3.9) although the mechanism whereby relocation and concentration in the kidney is achieved, is not clear.

In the liver cell, gold concentrates initially in the cytosol where complexes with non-specific binding proteins, small molecular weight compounds and a gold-induced protein are formed. Some gold diffuses across the hepato-biliary membrane but an extremely efficient concentration mechanism subsequently localises the metal within lysosomal vesicles which eventually discharge their contents into the bile. Biliary excretion therefore probably accounts for at least some of the faecal elimination of gold although losses into the gut from intestinal cells or with digestive secretions may also take place.

The role of lysosomes in concentrating gold in the liver cell allows speculation concerning the phagocytic activity of other cells and in particular, the macrophages. Given a similar exposure to gold cells with exceptional phagocytic ability will necessarily concentrate greater amounts of gold. In rheumatoid arthritis macrophages gravitate towards the inflamed joints and will thereby effectively scavenge and transfer gold in large concentrations to the loci of the disease where the palliative properties can be applied.

In the introduction to this Chapter the question was raised as to the importance of thiomalate in the metabolism of gold and it was suggested that the formation of complexes with simple thiol containing compounds would be central to some of the problems associated with chrysotherapy.

The evidence that has emerged indicated that gold has a greater impact upon the metabolism of thiomalic acid than *vice versa*. The addition of gold masks the active thiol group which otherwise is responsible for retention (in the muscle and hepatocellular cytosol) and the rapid clearance from plasma. It has been shown that aurothiomalate is totally absorbed from the muscle and that thiomalate and gold are fully separated.
intracellularly. It is therefore the plasma and cellular environments which expose gold to factors which may influence its metabolism. Association with proteins such as albumin - both in plasma and in cytosol which is the major quantitative factor although thiomalate (in plasma) and other thiols could provoke subtle changes in the availability of gold.

It is therefore to the distribution of gold between protein and non-protein fractions that further attention must be given. The concentration of albumin provides the opportunity for variation in gold distribution between individuals who are apparently similar with reference to dosage, total plasma gold concentration etc. At the same time other drugs and molecules which compete with gold for the same binding site can further shift the balance between bound and unbound gold. To ascertain whether there is a direct link between therapeutic and toxic responses and the plasma or tissue concentration of proteins and the gold distribution pattern has now become an important step.
CHAPTER FOUR

SOME EFFECTS OF GOLD UPON THE KIDNEY.

TRACE METAL INTERRELATIONSHIPS AND MECHANISMS OF TOXICITY
4.1 INTRODUCTION

Patients receiving chrysotherapy and animals given gold concentrate the metal in the kidney (Sections 1.5.2, 2.4.3, 3.3.3, and Table 3.4). The kidney is one of the organs in which gold may produce toxic damage and renal toxicity is the second most common side-effect associated with chrysotherapy (Table 1.5). All patients who are treated with gold should therefore be screened at regular intervals for the presence of urinary protein so that renal damage may be detected and treated at an early stage.

Gold nephropathy does not represent a single entity and renal damage induced by the metal may take different forms. Mild proteinuria is the main feature (106) although in a very few cases the urinary excretion of protein is much increased (greater than 5 g per 24 hours) and a typical nephrotic syndrome develops. (175) Microscopically a membranous glomerulonephritis is seen with thickening of the glomerular basement membrane, shown by immunofluorescent staining to contain immune complexes. (176) Gold-containing inclusion bodies are found in cells of the proximal tubule but not in those of the glomerulus. It is suggested (107) that acute tubular necrosis (which may be fatal) and tubular degeneration, are consequences of local gold toxicity while the glomerulonephritis represents an immunologically mediated response to gold.

Animals exposed experimentally to gold compounds also develop renal damage but the toxic manifestations are usually confined to the kidney tubule. (107) The inclusion bodies appear as dense, filamentous structures when viewed with the electron-microscope and gold can be detected by microprobe analysis. (156,157,177) They are observed in the proximal tubule cells and also in the interstitial macrophages. Thus toxic nephropathy in animals and in man has been well characterised morphologically but very few biochemical features have been detailed.

It is now appreciated that the low molecular weight binding proteins, metallothioneins, are involved in the homeostasis of essential elements, (178) offering control – e.g. over discharge of metal from the intestinal mucosal cell into blood, the uptake of metal from blood by the liver. However, it was as a consequence of its role in metal toxicology that metallothionein was first discovered. In this context thionein provides a protective detoxifying mechanism whereby the metal, e.g. mercury or cadmium is effectively rendered innocuous with the formation of an unavailable complex. (179)
When referring to hepatic metallothionein in the previous chapter (Sections 3.4 and 3.5) it was noted that the protein not only binds the cation which was responsible for induction of its synthesis but also other metals including the essential ions such as zinc and copper. Since it is the kidney that contains the greatest concentration of these proteins it is pertinent to consider whether gold-thionein might be responsible for alterations in the balance of trace-metals in this (or other) organ(s) and whether such changes might feature in gold toxicity.

Accordingly, experiments were conducted to determine whether significant levels of thionein were synthesised following exposure of rats to gold and if so, whether there were changes in the metal composition of body fluids and tissues. Assessment of tissue damage was made by examining the proteins in urine, by light and electron microscopy and by electron microprobe analysis of cellular structures.

4.2 METHODS

4.2.1 Experimental

(1) Fifteen Male Wistar Albino rats initially weighing 150 g were distributed randomly into three groups of five animals. Sodium aurothiomalate was administered by sub-cutaneous injection twice a week for four weeks at the following doses: Group I, 0 (0.1 ml 0.9% w/v saline was given), Group II, 1.5 mg/kg in saline, Group III, 7.5 mg/kg in saline. Two days after the final injection the rats were placed in glass metabolic cages and urine samples were collected for 24 hours. The animals were killed by cervical dislocation and the kidneys removed for determination of copper, zinc, iron, calcium and magnesium, isolation of cytosolic proteins and microscopic examination.

(2) Twelve Male Wistar Albino rats initially weighing about 150 g were divided into two groups each containing six animals. The test group were injected sub-cutaneously with 0.1 ml of sodium aurothiomalate solution (10 mg/ml) twice a week for five weeks. The second (control) group were similarly injected with 0.9% w/v saline.

Eighteen days after the start of the experiment (two days after the fifth injection) animals were placed in individual glass metabolic cages and their urine and faeces collected for 24 hours. Collection of excreta was repeated five days after the final (tenth) injection, immediately prior to killing the animals.
The rats were anaesthetised using pentobarbitone, blood removed by cardiac puncture and allowed to clot. The liver, kidney, spleen, heart, lung and testis were removed and weighed. The femur and a portion of the adjoining striated muscle were dissected from a rear leg.

(3) Biliary excretion of metals. Twelve Male Wistar Albino rats weighing about 200 g were divided into three groups. Group I (3 animals) were given sodium aurothiomalate 2.5 mg/kg by intraperitoneal injection two hours before cannulation of the bile duct and collection of bile. Group II (3 animals) were anaesthetised and biliary cannulation established 5-10 minutes before sodium aurothiomalate 2.5 mg/kg was administered by intravenous injection. Group III (6 animals) were the controls and received no aurothiomalate by either route. The procedures for the administration of sodium aurothiomalate and collection of bile were as described in Section 3.5.2.

4.2.2 Analytical

Metals. All metals were analysed by atomic absorption spectroscopy.

Reagents. Lanthanum chloride; 0.1% w/v La$^{2+}$.

TCA-TGA solution; 100 g trichloroacetic acid and 30 ml thioglycollic acid were dissolved in 166 ml hydrochloric acid and the total volume made up to one litre.

Nitric acid; aristar grade.

Perchloric acid Sp. Gr. 1.54; aristar grade.

Aqua Regia; 50 ml conc. nitric acid was carefully added to 50 ml conc. hydrochloric acid and mixed.

All glassware used in these analyses were soaked overnight in 10% v/v hydrochloric acid and thoroughly rinsed in distilled water.

Serum; Calcium and magnesium were measured after fiftyfold dilution in lanthanum chloride solution, samples for copper and zinc were diluted 1 + 4 in distilled water (180) and iron was measured after precipitation of the protein by addition of an equal volume of TCA-TGA solution. (181) Gold was measured as described in Section 2.2.1.

Urine; All metals were measured using methods of standard additions to calibrate the analyses. Samples were diluted as was found to be appropriate in order to achieve linear standard plots.
Faeces and tissues; Samples were prepared for analysis by acid digestion as described in Chapter Two (Section 2.3.1).

Bile; Copper, zinc, iron and gold were measured by pulse sampling flame atomic absorption taking 100 μl for each determination (Section 3.5.2).

Creatine was determined in serum and urine by an auto-analyser procedure using the alkaline picrate reagent.

Urine Proteins

Reagents. Sulphosalicylic acid, 3 g/100 ml.
Barbitone buffer, 0.06 M, pH 8.6; Sodium barbitone, 35.5 g, diethylbarbituric acid, 4.52 g and sodium acetate trihydrate, 26 g were dissolved in distilled water and the volume made to 4.0 litre.
Nigrosin stain; Nigrosin (1.0 g), glacial acetic acid, (150 ml), glycerol (50 ml), 0.1 M sodium acetate, (400 ml), and methanol (100 ml) were dissolved in distilled water and the total volume made to 1.0 litre.
Acetic acid 5.0% v/v.

Total protein concentration

Sulphosalicylic acid (3 ml) was added to 1.0 ml urine and the turbidity measured at 650 nm after 5 minutes using a Gilford 300 spectrophotometer. Turbidity was measured against a blank prepared from 1.0 ml urine plus 3.0 ml water.

Standards were prepared from dilutions of a freeze-dried serum sample of known protein concentration. Standards having concentrations in the range 0.1 to 1.5 g/l were used and turbidities determined as for the urine samples.

Cellulose acetate electrophoresis

Urine samples were separated without prior concentration. Conventional equipment and conditions were used. The cellulose acetate strips were stained in nigrosin solution for 2 to 3 minutes then destained in 5% acetic acid. The protein positions were revealed as black bands.
Isolation and characterisation of cytosolic proteins

Reagents. Ammonium formate buffer, 0.015 M, pH 8.0; A 1 M stock solution (6.306 g ammonium formate in 100 ml water) was prepared. Fifteen ml of this solution was diluted to 1 litre with distilled water and the pH adjusted to 8.0 with ammonium hydroxide.

Tris buffer, 0.01 M, pH 8.0; A 2 M stock solution (121.14 g Tris-(hydroxymethyl)-methylamine in 500 ml) was prepared. Fifty ml of this solution was diluted to 1 litre with distilled water and adjusted to pH 8.0 using 1 M hydrochloric acid.

Sephadex G75 in Tris buffer; the Sephadex suspension was prepared according to the manufacturer's (Pharmacia Ltd.) directions.

Approximately 2 g of kidney tissue was washed with ammonium formate buffer, blotted dry and weighed. The samples were added to 10 ml ice-cold ammonium formate buffer and homogenised as described for liver (Section 3.4.2). The homogenate was transferred to a centrifuge tube and an aliquot (200 μl) removed for determination of total protein. The cytosol was separated from cell debris and formed elements by centrifugation at 120,000 g for 60 minutes in an 8x35 ml angle rotor using an MSE Super Speed 65 centrifuge. The supernatant was transferred to a 50 ml round bottomed flask and the aqueous phase removed by lyophilization. Tris buffer (10 ml) was added to the freeze-dried residue and the flask placed in an ice-bath for ten minutes with occasional mixing to dissolve the contents. This solution was decanted into a plastic tube and centrifuged at 600 g for 10 minutes. A sample (0.5 ml) of the supernatant was removed for protein determination and the remainder separated by gel filtration against gravity.

A column (870x2.6 cm) of Sephadex G75 in Tris buffer was set up and the sample applied. An elution rate of 17 ml per hour was established and fractions (2.8 ml) were collected every 10 minutes after elution of an initial volume of 68 ml. A total of 120 fractions were collected. Absorbance of the eluate at 254 nm was continuously monitored. Gold, copper, zinc and iron were measured by atomic absorption spectroscopy. The samples were aspirated without any preparative procedure and readings.
were compared against those of standards diluted in Tris buffer. Quantitative measurements of protein concentrations were made using the Folin-Ciocalteu reagent of Lowry et al. (182)

Microscopy

Reagents. Buffered formalin; Sodium dihydrogen orthophosphate dihydrate, (4.5 g) and anhydrous disodium hydrogen orthophosphate, (6.5 g) were dissolved in distilled water. Formalin (40% formaldehyde, 40 ml) was added and the total volume made to 1 litre with water.

Ethanol.

Toluene.

Paraffin Wax.

Glutaraldehyde solution; E.M. grade stock solution, 25% w/v.

Cacodylate buffer, 0.2 M, pH7.4.

Sodium cacodylate (21.4 g) was dissolved in 400 ml distilled water. The pH was adjusted to 7.4 with dilute nitric acid and the total volume made to 500 ml.

Buffered glutaraldehyde. 16 ml stock glutaraldehyde was added to 50 ml cacodylate buffer and the volume made up to 100 ml with distilled water.

Osmic acid, 4 g/100 ml.

Buffered osmic acid. Cacodylate buffer and osmic acid were mixed together 1 + 1.

Propylene oxide.

Epon 812.

Tissue samples removed when the animals were killed were cut into small blocks and placed into appropriate fixatives.

For light microscopy fixing was in buffered formalin. Preparation of the tissue i.e. dehydration, wax embedding, section cutting, staining and counter-staining with eosin and haematoxylin, was carried out by conventional procedures. (183)

Material for electron microscopy (tissue blocks less than 0.5 mm cubes) were placed in glass tubes containing buffered glutaraldehyde at 4°C for two hours. The glutaraldehyde was replaced with 0.1 M cacodylate buffer
and washed for two hours. The tissues were counter-fixed for a further two hours by removal of the buffer and addition of buffered osmic acid. Samples were progressively dehydrated by immersion in solutions of ethanol (25%, 50%, 75%, 90%, absolute v/v). Each solution was left for at least 10 minutes and two changes of each solution were made. The samples were similarly taken through a mixture of ethanol/propylene oxide (1 + 1 v/v), propylene oxide and a mixture of propylene oxide/Epon 812 resin (1 + 1 v/v), and were then transferred to capsules containing Epon 812. The resin was polymerised at 60°C for 48 hours. Sections were cut and mounted for electron microscopy and stained with uranyl acetate and lead acetate.

A Jeol JEM 200 cx transmission electron microscope was used with additional facilities for scanning electron microscopy and electron microprobe analysis. (University of Surrey Micro Structural Studies Unit).

4.3 RESULTS

4.3.1 Evidence for Gold-induced Renal Changes

The structure of the kidney as observed by light microscopy is pictured in Figs. 4.1 and 4.2. Fig. 4.1 shows a section prepared from kidney taken from a control (saline-treated) rat and no pathological changes are evident. Fig. 4.2 shows the histology from a gold-treated (7.5 mg sodium aurothiomalate, sub-cutaneously, twice a week for four weeks) rat. Changes to the glomerulus were not found using haematoxylin and eosin stain. However, certain of the tubular cells had large pink coloured vacuoles which were localised along the medullary-cortex margin suggesting a situation primarily within the proximal tubules. (These vacuoles were identified at microscopy by an experienced pathologist (Prof. P. Grasso) but unfortunately do not show very clearly in the photographs).

Greater clarity of ultra structural and cellular architecture was appreciable from the electron microscopy (Figs. 4.3 - 4.6). Unfortunately sample fixation was not perfectly achieved and there was therefore some breakdown of structure in sections prepared from tissues of both the control and gold-exposed rats. Nevertheless it was possible to differentiate between them and to observe gold-induced changes in cell appearance.

Detail of cells of the proximal tubular region of the kidney from the control rat is shown at low (3,000) and high (10,000) magnification in
Fig. 4.1 Photomicrograph of normal kidney from rat given 0.9% w/v saline by sub-cutaneous injection twice a week for 5 weeks.

Fig. 4.2 Photomicrograph of a kidney from rat given sodium aurothiomalate (7.5 mg/kg) by sub-cutaneous injection twice a week for 5 weeks. The arrows indicate the densely staining vacuoles induced by treatment with gold.
Figures 4.3 to 4.6

Fig. 4.3 Electronphotomicrograph of normal kidney, 3,000 x magnification. Prepared from control rat given 0.9% w/v saline twice a week for 5 weeks. Relevant features are the villi (a) and the size and number of lysosomes (b).

Fig. 4.4 Electronphotomicrograph of normal rat kidney, 10,000 x magnification. The section shows a proximal tubular area with membranous invagination and signs of discharge of cellular contents into the lumen of the kidney tubule (a).

Fig. 4.5 Electronphotomicrograph of kidney from rat given sodium aurothiomalate (7.5 mg/kg by sub-cutaneous injection twice a week for 5 weeks, 3,000 x magnification. The section shows breakdown of the villi (a) and enlarged lysosomes which contain deposits of electron-dense material (b).

Fig. 4.6 Electronphotomicrograph of kidney from rat given sodium aurothiomalate, 10,000 x magnification. The electron-dense deposits with characteristic feathery outline are seen within a lysosome (a).
Figs. 4.3 and 4.4 while Figs. 4.5 and 4.6 show similar zones from a rat treated with sodium aurothiomalate. The particular features noted in the specimen from a rat given sodium aurothiomalate compared with the controls are the increased size of the lysosomes (Figs. 4.3 and 4.5), a breakdown of the villus structures at the tubule cellular-lumen margin (Figs. 4.3 and 4.5) and the presence of electron dense inclusions within the enlarged lysosomes (Figs. 4.4 but particularly 4.5).

These electron dense structures present in the renal tubule cells of the gold-treated rats were shown to contain gold by electron microprobe analysis. A spectrum obtained from one such analysis is given in Fig. 4.7. The spectrum shows the emissions characteristic of the fixing and staining metals - osmium (Mα, Lα, Lβ, Lγ), uranium (Mα, Lα) and lead (Mα, Lα) and also of the copper supporting grid (Kα). In addition the presence of gold was revealed from the Lα and Lβ emissions at 8.91 and 11.58 KeV. Analyses were also conducted at areas adjacent to, but distinct from the electron-dense bodies, and which had a similar visual background appearance. Gold emissions were not detected from these areas. It was noted (Table 4.1) that when the intensity of the copper Kα emissions at 8.04 KeV were compared at gold-rich or gold-free sites, greater amounts of copper were present in conjunction with the gold. Since a large proportion of the copper emission will have been derived from the supporting grid the increases represent a considerable enhancement of the in situ biological copper in association with gold.

Urine proteins

The excretion of protein in the urine (Table 4.2a) by the rats who received the higher dose of gold (Group III) was significantly increased (19.4 mg/24 hrs) compared with the controls (14.6 mg/24 hrs). On examination of unconcentrated urine samples by electrophoresis on cellulose acetate membranes, protein was detected only in urine samples from this same group. When compared with the separation pattern obtained for a sample of serum, the urine proteins were located between the α2 and β globulins. There was no albumin detected by this procedure.

The data obtained from the rats treated with sodium aurothiomalate for 5 weeks allowed the calculation of creatinine clearance rates (Table 4.2). The clearance for the control animals (1.99 ml/min) was not significantly different from that found for the rats who had received sodium aurothiomalate (1.58 ml/min).
Fig. 4.7 Electron microprobe analysis of electron dense inclusion body shown in Fig. 4.6.
Table 4.1 Electron Microprobe Analysis

<table>
<thead>
<tr>
<th>ENERGY</th>
<th>ELEMENT</th>
<th>SITE</th>
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<th>SITE</th>
</tr>
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<td></td>
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</tr>
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<td>9.71</td>
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<td>8.04</td>
<td>Cu</td>
<td>1629</td>
<td>1329</td>
<td>2517</td>
</tr>
</tbody>
</table>

Comparison of copper emission intensities in the presence of different amounts of gold in kidney (inner) - SECTION I, kidney (outer) - SECTION II and liver of rats given sodium aurothiomalate 7.5 mg/kg sub-cutaneously, twice a week for 4 weeks. The measurements suggest that at sites where there is localization of gold there are also increased amounts of copper.
Table 4.2  Excretion of protein in urine and the creatinine clearance of rats given sodium aurothiomalate by sub-cutaneous injection for four or five weeks.

(a) Protein (5 animals per group)

<table>
<thead>
<tr>
<th>Group</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg) twice per week for 4 weeks</td>
<td>0</td>
<td>1.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Urine protein mean mg/24 hrs</td>
<td>14.6</td>
<td>13.6</td>
<td>19.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Urine protein SD mg/24 hrs</td>
<td>2.9</td>
<td>5.06</td>
<td>3.38</td>
</tr>
</tbody>
</table>

<sup>a</sup>, significant difference from control group p = <0.05

(b) Creatinine clearance (6 animals per group)

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg) twice per week for 5 weeks</td>
<td>0</td>
<td>7.5</td>
</tr>
<tr>
<td>Creatinine clearance mean ml/min</td>
<td>1.99</td>
<td>1.58</td>
</tr>
<tr>
<td>Creatinine clearance SD ml/min</td>
<td>0.86</td>
<td>0.30</td>
</tr>
</tbody>
</table>
4.3.2 Biochemical Features of Gold and the Kidney

The 254 nm absorbance and trace metal elution profiles for the kidney cytosolic preparations are shown in Fig. 4.8. The protein (OD_{254 \text{nm}}) resolved into high molecular weight material (albumin, haemoglobin etc.) which eluted at or soon after the void volume - peaks Pr-1 and Pr-2 and low molecular weight compounds which appeared close to the total column volume - peaks Pr-4 and Pr-5. With the test sample from the gold-treated animals there was a small additional peak - Pr-3 which was found in the trough between the two extremes. This peak corresponded to an elution volume of 214 ml. (In other experiments with the same column using cytosolic preparations from cadmium-treated rats the OD_{254 \text{nm}} showed a large peak at this position. Cadmium-thionein, but not other metallothioneins, has an absorption maximum at 250 nm). The elution profiles for gold, copper, iron and zinc are also shown in Fig. 4.8.

Gold separated into two components Au-1 which appeared with the void volume and presumably corresponded to albumin bound metal and Au-2 which was coincidental with the Pr-3 peak at an elution volume of 214 ml. Most of the iron was associated with haemoglobin with smaller amounts co-eluting with the albumin (Pr-1) and low molecular weight compounds (Pr-4). Identical patterns were obtained for the test and control samples. With zinc and copper however there were different elution profiles for the two samples. With the material from the controls, zinc eluted in two positions, Zn-1 with albumin and Zn-3 together with Pr-4. The test sample had these two components but also a third, Zn-2 which coincided with Pr-3. The copper elution profile was also characterised by three components, Cu-1 with albumin, Cu-2 which eluted just after the haemoglobin (Pr-2) and Cu-3 which like Au-2 and Zn-2 was coincidental with the OD_{254 \text{nm}} peak, Pr-3. The copper concentrations in the eluate from the test sample were greater than in the control by a factor of about two in peaks Cu-1 and Cu-2 but about eight times in Cu-3.

The metal concentrations in the kidneys from the control and gold-treated rats are shown in Table 4.3. Chronic parenteral administration of sodium aurothiomalate produced changes in the total concentrations of copper and zinc, which reflected the results obtained from the separation of cytosolic components. The effects upon renal copper were dose related with increases of 350% and 550% compared with the controls. Increased zinc concentrations were also found but these were less dramatic and not dose dependent. No significant effect upon renal iron, calcium or magnesium were obtained.
Fig. 4.8 Gold, iron, zinc and copper (µg/ml) and absorbance (OD254nm) profiles of eluate from G75 Sephadex column (870 x 2.6 cm) after application of kidney cytosolic preparations from a rat given sodium aurothiomalate (7.5 mg/kg) or a rat given 0.1 ml saline (0.9% w/v) sub-cutaneously, twice a week for 5 weeks.

Fractions of eluate from the column show different peaks for gold, iron, zinc, copper and protein absorbance. Each metal and protein has its own characteristic absorbance profile.
<table>
<thead>
<tr>
<th>Group</th>
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<th>Zn</th>
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<tbody>
<tr>
<td>control</td>
<td>mean</td>
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<td>28.53</td>
<td>92.55</td>
<td>61.38</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>1.28</td>
<td>0.56</td>
<td>5.82</td>
<td>8.46</td>
</tr>
<tr>
<td>1.5 mg/kg</td>
<td>mean</td>
<td>28.38(^a)</td>
<td>35.73(^b)</td>
<td>89.38</td>
<td>56.50</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>3.53</td>
<td>2.74</td>
<td>6.39</td>
<td>2.78</td>
</tr>
<tr>
<td>7.5 mg/kg</td>
<td>mean</td>
<td>44.55(^a) (^c)</td>
<td>33.68(^b)</td>
<td>83.10</td>
<td>59.35</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>2.95</td>
<td>2.52</td>
<td>14.50</td>
<td>2.46</td>
</tr>
</tbody>
</table>

Sodium aurothiomalate 1.5 mg/kg or 7.5 mg/kg given by sub-cutaneous injection twice a week for four weeks. Controls similarly given 0.9% w/v sodium chloride.

- \(^a\) significant difference compared with control group p = \(<0.001\)
- \(^b\) significant difference compared with control group p = \(<0.01\)
- \(^c\) significant difference between the gold treated groups p = \(<0.001\)
4.3.3 Metal Interactions in Body Fluids and Tissues

Weights

The body and organ weights of the control and gold-treated rats after administration of sodium aurothiomalate for 5 weeks, are shown in Table 4.4. No significant differences were found in any parameter between the two groups.

Serum and excreta

The serum and urinary metal and creatinine concentrations and the excretion of metals into the faeces are given in Table 4.5. The mean serum copper concentration (15.28 µmol/l) was significantly lower in the gold-treated rats than in the controls (17.53 µmol/l). After the administration of gold for three weeks, the urinary excretion of magnesium and the faecal elimination of copper, zinc and iron were significantly decreased compared with the control animals. There were no significant differences in any of the other analytes measured in these samples nor in those collected after five weeks although consistent trends could be observed. At five weeks after the start of the experiment most of the mean faecal measurements were lower in the gold-treated animals than in the controls but as a consequence of one sample with a very large weight (possibly representing more than 24 hours) the standard deviation concentrations were larger (Table 4.5c).

Tissues

The most dramatic change in metal concentrations (Table 4.6) were found in the kidney where copper and zinc concentrations were increased (as was found in the previous experiment - Table 4.3) by 560% and 124% respectively. Increased copper concentrations were also found in muscle tissue and in the samples of bone. Other significant changes in elemental composition were the increased iron content of testis and muscle and an increase in the calcium in spleen. In all these tissues the effect of gold upon metal concentration was accumulative.

4.3.4 Biliary Metals

The secretion of copper, iron and zinc into the bile in control rats and in animals given gold by intravenous injection are shown in Figs. 4.9 - 4.11 respectively.
Table 4.4 Effect of chronic gold administration upon body and organ weights in rats given sodium aurothiomalate.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Weight (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Total body weight</td>
<td>386 ± 34</td>
</tr>
<tr>
<td>Liver</td>
<td>14.7 ± 1.9</td>
</tr>
<tr>
<td>Kidney (both)</td>
<td>2.3 ± 0.16</td>
</tr>
<tr>
<td>Heart</td>
<td>1.01 ± 0.08</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.90 ± 0.15</td>
</tr>
<tr>
<td>Testis (one)</td>
<td>1.68 ± 0.15</td>
</tr>
<tr>
<td>Lung</td>
<td>1.47 ± 0.18</td>
</tr>
</tbody>
</table>

Test group given sodium aurothiomalate, 7.5 mg/kg by sub-cutaneous injection, twice a week for 5 weeks.

Controls given 0.9% w/v sodium chloride (0.1 ml) by sub-cutaneous injection, twice a week for 5 weeks.

Each group contained 6 animals.
Table 4.5 Concentration of metals and creatinine in serum and urine and concentration of metals in faeces of gold treated rats (7.5 mg/kg sodium aurothiomalate) and their controls (0.9% w/v sodium chloride). Compounds given by subcutaneous injection twice a week for 5 weeks. (6 animals in each group).

### 4.5a

<table>
<thead>
<tr>
<th></th>
<th>Ca mmol/(\lambda)</th>
<th>Mg</th>
<th>Cu (\mu)mol/(\lambda)</th>
<th>Zn (\mu)mol/(\lambda)</th>
<th>Fe</th>
<th>Au</th>
<th>Creatinine (\mu)mol/(\lambda)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S E R U M 5 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL ANIMALS</td>
<td>mean</td>
<td>2.12</td>
<td>1.09</td>
<td>17.53</td>
<td>19.20</td>
<td>33.58</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.53</td>
<td>0.11</td>
<td>1.45</td>
<td>0.76</td>
<td>6.60</td>
<td>-</td>
</tr>
<tr>
<td>TEST ANIMALS</td>
<td>mean</td>
<td>2.25</td>
<td>1.09</td>
<td>15.28(^a)</td>
<td>18.98</td>
<td>30.85</td>
<td>3.45</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.26</td>
<td>0.09</td>
<td>0.79</td>
<td>1.82</td>
<td>2.77</td>
<td>0.58</td>
</tr>
</tbody>
</table>

### 4.5b

<table>
<thead>
<tr>
<th></th>
<th>Ca mmol/mmol</th>
<th>Mg</th>
<th>Cu (\mu)mol/mmol</th>
<th>Zn creatinine</th>
<th>Fe</th>
<th>Au</th>
<th>Creatinine mmol/(\lambda)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U R I N E 3 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL ANIMALS</td>
<td>mean</td>
<td>0.29</td>
<td>1.98</td>
<td>2.70</td>
<td>0.82</td>
<td>0.89</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.24</td>
<td>1.81</td>
<td>1.61</td>
<td>0.56</td>
<td>0.76</td>
<td>-</td>
</tr>
<tr>
<td>TEST ANIMALS</td>
<td>mean</td>
<td>0.27</td>
<td>0.56(^b)</td>
<td>2.09</td>
<td>0.57</td>
<td>0.50</td>
<td>1.54</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.09</td>
<td>0.64</td>
<td>0.44</td>
<td>0.08</td>
<td>0.17</td>
<td>0.76</td>
</tr>
<tr>
<td>U R I N E 5 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL ANIMALS</td>
<td>mean</td>
<td>0.52</td>
<td>1.04</td>
<td>1.78</td>
<td>0.70</td>
<td>0.53</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.63</td>
<td>0.57</td>
<td>1.64</td>
<td>0.55</td>
<td>0.41</td>
<td>-</td>
</tr>
<tr>
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<td>0.40</td>
<td>0.54</td>
<td>1.49</td>
<td>0.48</td>
<td>0.30</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.19</td>
<td>0.64</td>
<td>0.33</td>
<td>0.08</td>
<td>0.07</td>
<td>0.83</td>
</tr>
</tbody>
</table>
# FAECAL METALS

**µg/24 hrs (mg/24 hrs*)**

<table>
<thead>
<tr>
<th></th>
<th>Ca</th>
<th>Mg</th>
<th>Cu</th>
<th>Zn</th>
<th>Fe</th>
<th>Au</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3 weeks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL ANIMALS</td>
<td>mean</td>
<td>11.98</td>
<td>5.03</td>
<td>70.58</td>
<td>370.8</td>
<td>1196</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>1.62</td>
<td>0.66</td>
<td>4.18</td>
<td>31.9</td>
<td>264</td>
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<tr>
<td>TEST ANIMALS</td>
<td>mean</td>
<td>10.70</td>
<td>4.49</td>
<td>61.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>299.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>888&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>2.87</td>
<td>0.82</td>
<td>8.91</td>
<td>52.0</td>
<td>179</td>
</tr>
</tbody>
</table>

| **5 weeks** |     |     |     |     |     |     |
| CONTROL ANIMALS | mean | 17.87 | 7.72 | 97.62 | 518.3 | 1360 |
|               | SD  | 5.16 | 1.79 | 14.92 | 108.0 | 293  |
| TEST ANIMALS  | mean | 20.17 | 9.29 | 99.60 | 515.5 | 1471 |
|               | SD  | 7.09 | 4.07 | 34.97 | 190.3 | 522  |

significant difference  

a = <0.05  
from control  
b = <0.01
Table 4.6  Concentration of metals in tissues of rats given 7.5 mg sodium aurothiomalate/kg sub-cutaneously twice a week for five weeks and their controls (6 animals in each group). *Calcium and magnesium concentration in bone = mg/g wet weight.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Group</th>
<th>Cu</th>
<th>Zn</th>
<th>Fe</th>
<th>Ca</th>
<th>Mg</th>
<th>Au</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>Control mean</td>
<td>7.97</td>
<td>24.43</td>
<td>71.86</td>
<td>55.06</td>
<td>193.6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>2.99</td>
<td>2.23</td>
<td>10.95</td>
<td>3.65</td>
<td>9.34</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Test mean</td>
<td>44.66&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.38</td>
<td>53.92</td>
<td>193.8</td>
<td>143.4</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>7.19</td>
<td>2.45</td>
<td>15.01</td>
<td>3.34</td>
<td>15.88</td>
<td>10.7</td>
</tr>
<tr>
<td>Liver</td>
<td>Control mean</td>
<td>5.32</td>
<td>42.81</td>
<td>160.20</td>
<td>32.76</td>
<td>227.2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.48</td>
<td>8.26</td>
<td>30.40</td>
<td>4.23</td>
<td>39.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Test mean</td>
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<td>180.60</td>
<td>34.28</td>
<td>232.8</td>
<td>17.5</td>
</tr>
<tr>
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<td>34.64</td>
<td>0.83</td>
<td>13.3</td>
<td>3.6</td>
</tr>
<tr>
<td>Spleen</td>
<td>Control mean</td>
<td>2.08</td>
<td>25.75</td>
<td>617.2</td>
<td>25.01</td>
<td>265.4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.72</td>
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<td>139.0</td>
<td>3.19</td>
<td>68.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>2.60</td>
<td>32.42</td>
<td>854.0</td>
<td>32.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>303.6</td>
<td>14.2</td>
</tr>
<tr>
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<td>6.45</td>
<td>223.0</td>
<td>4.65</td>
<td>84.6</td>
<td>1.8</td>
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<td>5.73</td>
<td>16.69</td>
<td>100.34</td>
<td>6.36</td>
<td>186.9</td>
<td>-</td>
</tr>
<tr>
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<td>SD</td>
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<td>4.25</td>
<td>11.43</td>
<td>1.39</td>
<td>35.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>6.93</td>
<td>18.47</td>
<td>116.69</td>
<td>6.97</td>
<td>198.9</td>
<td>9.8</td>
</tr>
<tr>
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<td>1.05</td>
<td>2.39</td>
<td>23.99</td>
<td>1.26</td>
<td>34.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Tissue</td>
<td>Group</td>
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<td>Zn</td>
<td>Fe</td>
<td>Ca</td>
<td>Mg</td>
<td>Au</td>
</tr>
<tr>
<td>--------</td>
<td>---------</td>
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<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>Lung</td>
<td>Control</td>
<td>mean</td>
<td>2.37</td>
<td>22.09</td>
<td>172.9</td>
<td>41.58</td>
<td>171.4</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
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<td>17.68</td>
<td>150.6</td>
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<td>46.8</td>
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<td>14.86</td>
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</tr>
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<td>4.74</td>
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<td>13.35</td>
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</tr>
<tr>
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<td>Test</td>
<td>mean</td>
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<td>39.17</td>
<td>46.96^a</td>
<td>16.76</td>
<td>111.09</td>
</tr>
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<td>6.98</td>
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<td>1.47</td>
<td>29.53</td>
<td>14.21</td>
</tr>
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<td>Control</td>
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<td>22.18</td>
<td>137.60</td>
</tr>
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<td>5.55</td>
<td>1.60</td>
<td>7.13</td>
<td>27.3</td>
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</tr>
<tr>
<td></td>
<td>Test</td>
<td>mean</td>
<td>2.57^b</td>
<td>16.39</td>
<td>56.45^c</td>
<td>22.43</td>
<td>150.0</td>
</tr>
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<td>SD</td>
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<td>4.76</td>
<td>5.05</td>
<td>7.16</td>
<td>37.7</td>
<td>4.08</td>
</tr>
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<td>Control</td>
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<td>92.97</td>
<td>27.69</td>
<td>67.76*</td>
<td>1.75*</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.22</td>
<td>9.81</td>
<td>5.58</td>
<td>6.46</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Test</td>
<td>mean</td>
<td>2.30^a</td>
<td>101.57</td>
<td>30.14</td>
<td>71.13*</td>
<td>1.69*</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.42</td>
<td>9.67</td>
<td>5.18</td>
<td>12.12</td>
<td>0.17</td>
<td>0.49</td>
</tr>
</tbody>
</table>

significant difference
a  p = <0.05
b  p = <0.01
c  p = <0.001
As is shown with the control rats there are time-related changes in the rate of excretion of copper and iron with a gradual decrease in the amount of copper appearing in bile whereas the secretion of iron increased during the period of collection. Concentrations at the start of an experiment (controls or pre-intravenous injection) were; copper $0.63 \pm 0.09 \, \mu g/ml$, iron $0.48 \pm 0.07 \, \mu g/ml$ and zinc $0.068 \pm 0.01 \, \mu g/ml$ (results are mean ± standard deviations).

4.4 DISCUSSION

In this series of experiments sodium aurothiomalate was chronically administered to rats at doses of 1.5 to 7.5 mg/kg for periods of 4 or 5 weeks or given acutely by intravenous injection at a dose of 2.5 mg/kg. The results from the chronic exposure experiments confirmed that the gold compound has an effect upon renal structure and function and for the first time have demonstrated an influence upon trace metal disposition in a range of body tissues and fluids.

When compared with the control animals, the urinary excretion of protein was significantly increased in the animals given 1.5 mg/kg of the gold compound. The proteins present in the urine were not identified but on cellulose acetate electrophoresis the main components (qualitatively) were found in the α2 to β region (when compared against a diluted sample of serum). This proteinuria was very similar to that described following administration of cadmium (184,185) which is known to be caused by tubular dysfunction and is also similar to the tubular type proteinuria described in man.

Furthermore, the absence of albumin on the electrophoretograms and the normal creatinine clearance in the gold-treated rats suggests that there was no disturbance of renal glomerular function.

Evidence obtained by microscopy was also consistent with the occurrence of renal tubular rather than glomerular damage. The picture suggests the passage of a toxic agent through the glomerulus into the filtrate with re-absorption into the cells of the proximal tubule. The features which support this mechanism are the degeneration of villi and the enlarged lysosomes observed with the electron microscope (Fig. 4.5) and the appearance of large vacuoles within the cells when seen by light microscopy (Fig. 4.2). These signs are also observed with other tubular nephrotoxic compounds such
Fig. 4.9 Secretion of copper into the bile in control rats (n = 6), and in rats (n = 3) given sodium aurothiomalate (2.5 mg/kg) by intravenous injection. Results show mean ± standard deviation.
Fig. 4.10  Secretion of iron into the bile in control rats (n = 6) – – – –, and in rats (n = 3) given sodium aurothiomalate (2.5 mg/kg) by intravenous injection – – – –. Results show mean ± standard deviation.
Fig. 4.11  Secretion of zinc into the bile in control rats (n = 6) — , and in rats (n = 3) given sodium aurothiomalate (2.5 mg/kg) by intravenous injection — . Results show mean ± standard deviation.
as cadmium which are believed to be transported through the kidney in this way. At the same time there were no ultra-structural changes found in the glomerulus, either by light or electron microscopy which were indicative of membranous glomerulonephritis. In addition, the large electron dense inclusion bodies (Fig. 4.6) shown by microprobe analysis to contain gold (Fig. 4.7), were found only within the tubular region of the kidney.

Vacuolization observed in proximal tubular cells by light microscopy was remarked upon by Antonovych (107) although in this report the species exposed, the gold compound administered and the conditions of exposure were not stated. This author also claimed that the primary site of gold deposition was within the mitochondria which eventually show signs of degeneration. (107) While not readily apparent in Figs. 4.5 and 4.6 the sections of kidney from rats treated with sodium aurothiomalate contained mitochondria of normal appearance and gold was not detected in them by electron microprobe analysis when a series were examined. Antonovych therefore possibly used a more extreme course of administration to induce such changes.

The gold-containing electron dense bodies found within lysosomes of the proximal tubular tissue (Figs. 4.5 and 4.6) were identical in appearance with those described by Ghadially (157) and others. These dense granules with a feathery outline have been found at many sites in several species and, with the exception of those formed on exposure to colloidal gold, assume a consistent morphology. Since gold at high concentrations is known to inhibit lysosomal enzymes (Chapter 1) accumulation of the metal within these organelles gives support to the hypothesis that chrysotherapy is effective by an action upon the activity of lytic enzymes. The microscopy also revealed that in animals where a specific disturbance in renal function was demonstrated (Section 4.3.1) a coincidental localisation of gold also occurred.

From this series of experiments there emerged a consistent pattern with an association between gold and trace metal concentrations, particularly with copper (Fig. 4.12).

In his lecture at the 1977 Symposium on the Clinical Chemistry and Chemical Toxicology of Metals, Schwarz (186) illustrated a point relating to trace
Fig. 4.12 Interactions found to occur between gold (sodium aurothiomalate) and other metals in the rat. (All effects were significant at levels of 5% or less).
metal interactions using a chart prepared in 1953. He noted that such a chart similarly prepared in 1977 to demonstrate the interactions then known to exist would be far too complex to comprehend. In the five years since that observation was made by Schwarz, examples of the importance of biological interactions between metals have continued to be described with increasing frequency.

Interactions between metals are important in the metabolism and distribution of essential elements at various sites throughout the body. Intestinal transport of iron is inhibited by cadmium (187) and similar, reciprocal effects have been described among other elements e.g. copper, zinc, mercury and lead. (188-192) Other sites where competition between metals is a feature of normal metabolism include excretion into the faeces (193) and uptake into cells, e.g. of the liver. (194) Metal interactions are also important in toxicity. Excessive exposure to copper interferes with iron metabolism, decreasing tissue concentrations and producing a situation of iron deficiency anaemia. (195) Cadmium alters not only the tissue concentrations of trace elements but also major elements such as calcium. (196) Changes in the serum concentrations of trace elements, apparently unrelated to the aetiology of the disease, have been noted in many pathological conditions and may be measured in order to monitor the progress of the patient. (197)

In addition to changes in tissue metal concentrations which are consequent upon metal interactions at the intestine, modulation of tissue metal composition has been found in animals treated with e.g. mercury (198,199), bismuth (199) and cadmium (199-202). In these reports, changes in the renal metal concentration has been a regular response with an increase in copper the most significant finding. Recently it has been demonstrated that aurothioglucose administered to mice on 10 occasions during three weeks also produced increases in kidney copper and in renal and hepatic zinc concentrations. (203) These animals received very large doses of the gold compound (50, 200 and 400 mg/kg) compared with the doses of aurothiomalate given in the experiments reported above. With the mice, the changes in tissue zinc were only found in animals which received the highest dose of aurothioglucose whereas the increases in renal copper were also dose related and occurred in all three groups.

Types of metal interactions and possible mechanisms whereby they may be effected were considered by a Task Group on Metal Interaction at a meeting.
concerning Factors Influencing Metabolism and Toxicity of Metals. (204)
The Group concluded that metals may react to produce synergistic, antagonistic or entirely new types of effects. Possible mechanisms of these interactions were summarised as:

(a) formation of metal complexes which reduce bio-availability,
(b) interchange of metals bound to proteins such as carrier proteins, plasma albumin, enzymes and metallothionein,
(c) attenuate cellular reactivity to a subsequent exposure,
(d) induction of metal-binding proteins.

Some reactions however do not fit into a situation with just one mechanism (205) and it is necessary to propose interactions occurring at different sites and involving more than one of the above mechanisms.

In seeking to explain how exposure to metals such as mercury, cadmium etc. produce an increase in renal and hepatic concentrations of copper and other metals authors (196,198,202,203) have presumed a mechanism involving metallothionein. The proteins isolated from tissues of cadmium or mercury-treated animals have been shown also to bind zinc and copper. (179) It has been argued therefore that the alterations in tissue metal content are secondary to the synthesis of and sequestration by, metallothionein. Evidence to substantiate this hypothesis (which invokes mechanisms (b) and (d) above) by examining cytosolic metal binding protein and total tissue concentrations at the same time, has however been singularly lacking.

The results presented here from rats treated with sodium aurothiomalate (Figs. 4.8 - 4.11, Tables 4.3 - 4.6) indicate changes in trace metal disposition which are similar to those realised in animals exposed to other metals and also to aurothioglucose. Changes in zinc, copper, iron, manganese, calcium and magnesium concentrations have been described in liver, spleen, thymus, lung, heart and bone. (196,203,206,207) Alterations in metal concentrations in these studies were variable in amplitude and direction depending upon size, route and length of exposure, species and organ examined. Furthermore, increases in renal copper were also produced in a range of species (rat, mouse, sheep) with varying routes of administration. (198-200,203,208) In some of these studies the renal concentration of other metals was also determined with increases in zinc, iron,
magnesium, calcium and manganese noted. (203,207,208) The increase in renal copper was always the most sensitive phenomenon. In the experiments reported here it has been shown that aurothiomalate produced increased renal copper and zinc concentrations and at the same time, in the same animals, there was induction of the synthesis of metal-binding protein. This represents one of the few experiments in which a close association has been shown between these two independently observed responses to the administration of any metal.

Low molecular weight gold binding proteins have been isolated from liver and kidney of experimental animals treated with aurothiomalate (209-211) or with chloroauric acid (212,213) although not all attempts have been successful. (214) In addition it has been demonstrated that aurothiomalate will bind \textit{in vitro} to thionein synthesised in response to cadmium (211) and zinc. (214)

Two possibilities must therefore be borne in mind in any consideration of the results obtained for the cytosolic binding protein experiments. The protein may have been a true gold-thionein, synthesised in response to the challenge of the aurothiomalate. Alternatively, there may have been binding of gold to a pre-existing or simultaneously produced thionein which was present in response to some other stimulation. For two reasons the latter mechanism can probably be discounted.

1. There were quite distinct differences between the results in the control and test animals. The copper and zinc elution profiles and the \( \text{OD}_{254 \text{ nm}} \) readings showed evidence for a low molecular weight binding protein only in the test samples and not in those from the control. If the induction of metallothionein synthesis had been consequent upon some extraneous stimulus e.g. handling, receiving injections etc. which have been demonstrated to promote zinc thionein production (215-217) or upon contamination of the diet by another metal, similar results would have been obtained in both groups.

2. The metal which had the greatest binding to the low molecular weight protein was copper. Effective isolation and demonstration of copper-thionein requires anaerobic conditions to avoid loss of the metal from the ligand. (158) The copper remained in association with this particular protein even though an anaerobic environment was not established. It can be inferred therefore that the protein was not copper-thionein to which the gold also became bound. The relatively low levels of zinc compared
with copper and gold which were found together with the protein and the
minimal OD$_{254}$ readings also suggest that the protein was not a zinc-
thionein nor a cadmium-thionein.

It is improbable therefore that the low molecular weight protein induced
in the rats given sodium aurothiomalate is anything other than gold-
thionein. It can then be argued that induction of gold-thionein synthesis
and the binding to it of copper and zinc (two of the mechanisms for metal
interactions which were proposed by the Task Group (204)) operated to effect
the accumulation of gold, copper and zinc in the kidney and liver of these
animals.

Synthesis of cadmium-thionein has been shown to occur in several other
organs also (e.g. spleen, thymus, heart, brain, testis). (207,218) Thus
the increased metal concentrations observed in most of the rat tissues
(Table 4.6) could be a consequence of gold-thionein formation at a similar
wide range of sites. Sequestration of copper and zinc into gold-thionein
could be further responsible for the reduced concentrations of metals
measured in serum and urine (Table 4.5).

It was of particular interest to find that the electron dense deposits
which contained gold also appeared to contain increased amounts of copper
(Table 4.1). While this observation requires the examination of many more
animals and measurements in additional numbers of deposits within each
section to confirm a relationship between these metals, it was a consistent
feature of all those which were analysed.

Microprobe analysis of these gold bodies has been attempted by Ghadially
(156,157) and Tubbs et al. (177) The latter workers examined tissue
removed on renal biopsy from a patient with gold induced glomerulonephritis
and detected copper (when using a nylon support grid) and calcium.
Ghadially has performed more extensive analyses with synovial membrane,
articulart cartilage, liver, kidney, spleen, bone marrow and skin of rabbits
and rats. Gold inclusion bodies were found at all these sites and he was
able to find phosphorus in all cases, usually sulphur also and sometimes
 traces of calcium. Ghadially also reported that the composition of bodies
did not alter over the course of several months.

Such deposits imply that the gold has undergone considerable transformation
since gold(I) thiol and phosphine complexes are very soluble (Chapter 1).
The presence of sulphur and phosphorus may represent other lysosomal contents.
but could also suggest that an insoluble sulphide or phosphate complex is formed with gold. Alternatively, the incorporation of cations such as copper may be instrumental in the production of an insoluble mixed metal cluster. Thionein aggregation in the presence of copper has been reported (219) and if copper and cadmium are administered simultaneously, incorporation of cadmium is reduced with resultant enhanced toxicity. (220) Aggregated thionein would have a reduced capacity for metal binding which could explain this finding. (220) An altered protein-metal complex, particularly where aggregation also occurs would be expected to be removed to lysosomes.

It is evident that gold distributes among several components within cytosol. The elution profiles obtained from the rat kidney high speed supernatant (Fig. 4.8) and the data from liver and kidney separations (153,210,211) demonstrated that gold binds to high and low molecular weight compounds with approximately 20-30% of cytosolic metal associated with the thionein-like protein. Other metals e.g. copper and zinc similarly bind to more than one cytosolic component. Metal interactions could therefore be expected to involve other proteins in addition to metallothionein.

Following intravenous injection of sodium aurothiomalate the interaction between gold and copper in the liver cell, which influences the secretion of copper into bile, occurs within too short a time to be consequent upon sequestration into newly synthesised metallothionein (Fig. 4.9). The results are more consistent with a mechanism involving competition between the metals for a shared binding site. Copper entering the liver cell becomes incorporated into several proteins including the plasma transporting protein caeruloplasmin. Radiolabelling experiments show that once synthesised, caeruloplasmin is very rapidly removed from the hepatocyte into the circulation and that very little enters the bile. (221)

Cytosolic cuproproteins occur with molecular weights of about 150,000 (caeruloplasmin), 31,000 and 11,000. Injected radioactive copper enters the low molecular weight fraction (probably a thionein) but is soon transferred to the molecular weight 31,000 compound (superoxidedismutase) which coincides with the position of most of the stable copper in liver cytosol. In gel chromatography experiments, copper in bile was found in two components (221,222) but Frommer (222) suggested that the high molecular weight peak (greater than 800,000) was an artefact which was abolished when bile salts
were included in the eluting solution. It is suggested therefore that biliary copper occurs attached to one molecule (or molecular aggregate) of about 5,000 molecular weight. (223) This protein does not appear to have been characterised.

The proposition that copper could be easily displaced from a binding site is supported by the following data.

1. Cytoplasmic copper-metallothionein concentrations are increased in primary biliary cirrhosis. At least during the early stages of the disease the copper is very labile and can be removed using penicillamine. (223) This is in contrast to e.g. cadmium-thionein where the metal is resistant to mobilization.

2. The transfer of hepatic copper from thionein to superoxide dismutase (221) presents an opportunity for displacement.

3. Copper added to bile \textit{in vitro} distributes with the same pattern as is found for the endogenous metal. (222) This suggests that the copper is added onto the protein and is not incorporated as an integral part of the molecule as is found with caeruloplasmin.

Gold could be envisaged as competing for or displacing copper from the protein at any of these sites and influence the transport of copper into the bile.

Whether there exists an association between toxicity and the changes in trace metal disposition which follow from the administration of gold will be considered in the final chapter.
CHAPTER FIVE

DISCUSSION AND CONCLUSIONS
It is evident from the introductory discussion that the possible mechanisms of action of gold are extremely complex with the involvement of several different sites (Fig. 1.4). The overall aim of this project was to investigate some of the biochemical responses to the administration of sodium aurothiomalate in order that mechanisms responsible for the beneficial and toxic sequelae of chrysotherapy might be identified. With an awareness of the metabolic reaction involved, improved techniques for the management of patients receiving chrysotherapy could then be anticipated. To this end, investigations were made in patients and experimental animals, of acute and chronic effects of gold with particular emphasis on the concentration of the metal in body fluids, the tissue and subcellular disposition, the routes of elimination and interactions with essential trace-metals.

The first objectives studied were the serum and urine gold concentrations in patients with rheumatoid arthritis undergoing chrysotherapy. Preliminary reports demonstrated that a wide range of serum gold concentrations are produced following administration of a standard dose of a gold drug. Different body size is one parameter which will influence the plasma responses but the spread of concentrations, e.g. 2.54-14.8 \mu mol/l (Table 2.12) suggests that other factors are also involved.

The results in the retrospective and prospective studies showed that there was no association between serum or urinary gold concentrations and the therapeutic response. Patients whose rheumatoid condition remained unaltered had serum gold levels within the same range as did those who showed moderate or marked improvement (Table 2.3). Similarly, during the investigation of maintenance chrysotherapy, differences in clinical response were not found between patients with different concentrations of serum gold as a consequence of receiving gold at 2-weekly or 4-weekly intervals (Tables 2.9-2.11).

These results, which are in agreement with those recently published by other authors (80,81,100,131,132,140,141) are disappointing in that contrary to the hypothesis of Lorber (86) it is not possible to adopt the concept of a therapeutic blood concentration and to improve therapy by manipulation of the dose so that this level is maintained. It is appropriate therefore to look closely at the features involved in gold metabolism (as demonstrated by the experiments with animals) and assess the importance of these via a via response. The points to be considered include the rate of absorption from
the site of injection, compartmentalization of plasma gold, rates of tissue uptake and excretion, transfer of 'active' gold species to sites of action and the sensitivity of target sites, (Fig. 5.1).

Recovery of almost 100% of administered carbon-14 from rats given sodium (1,4-\(^{14}\)C)aurothiomalate (Figs. 3.7, 3.8) and the retention of very little radioactivity in muscle 24 hours after injection demonstrates that there is complete absorption of the administered drug into the circulation. Assuming that assimilation occurs to the same extent from subsequent injections it cannot be argued that variable rates of absorption account for the spectrum of clinical response.

Within the plasma there are an abundance of compounds shown to be relevant to further metabolism of sodium aurothiomalate. Therefore it is the circulating gold compound which is particularly vulnerable to individual, patient differences. The in vitro binding experiments demonstrated that several gold species will exist in plasma. The most abundant of these was an albumin-Au complex (Fig. 3.26) which is consistent with the measurements made on serum from gold-treated patients. (76,90,91) These experiments also showed that not all the gold was lost from thiomalate and that some aurothiomalate continues to exist, both as albumin bound and non-bound compound. Since binding of the metal to albumin is particularly avid (Table 3.7) (162,163), it is not unreasonable to presume that the non-protein bound fraction will represent, or include, the 'active' gold pool. Hypoalbuminaemia and low plasma albumin-globulin ratios are well recognised features of rheumatoid arthritis (64,65,224) and the size of the non-bound gold pool will therefore be greater in patients than in normal subjects. Moreover, concentrations of albumin will not be identical in all patients (e.g. 30.6-38.9 g/\(\ell\) quoted in reference 64) with an effect therefore upon the size of the free pool. A consequence of such an inference is that gold-albumin ratios may correlate more significantly with clinical response than does just the total gold concentration.

Albumin was shown to be responsible for separation of the gold drug also into aurothiomalate and R-Au species, i.e. for the removal of gold from the thiomalate (Figs. 3.21 - 3.26). This transformation however will probably be further influenced by thiol compounds within the plasma. Reactions of metals and thiol compounds have been recognised for a number of years. The toxicity of lead and mercury for example has been attributed to the binding with -SH groups of enzymes and an inhibitory effect upon catalytic activity. (225)
Fig. 5.1 Representation of different features involved in gold metabolism.

- Site of injection → Rate of absorption → Concentration → Compartmentalization → Inactive fraction → Active fraction → Effects of albumin (thiols, carrier molecule, other drugs, other metals) → Transfer to target tissues → Non-specific deposition → Rates of tissue uptake → Clinical improvement → Sensit of target tissue → Toxicity → Additional factor → Response in target tissues
Binding of metals to smaller molecular weight thiol compounds has also been recognised with the special role of thioneins in metal metabolism referred to in earlier chapters. A proportion of circulating zinc (and other trace-metals) is made up of complexes with amino-acids and small peptides. This pool assumes special significance in circumstances where the concentration of these metals is increased e.g. muscle catabolism, since urinary excretion is then considerably increased. (226)

More recently, small molecular weight thiol compounds have been shown to be involved in cellular uptake, organ distribution and excretion of metals. Alterations to the cellular concentration of non-protein sulphydryl groups e.g. by diethylmaleate, have significant effects upon the uptake of mercury and subsequent development of toxicity, in rats. (227,228) The finding of urinary metabolites from rats given carbon-14 labelled aurothiomalate or thiomalic acid which may have included complexes of aurothiomalate with cysteine and thiomalate with glutathione (Table 3.2) is consistent with an hypothesis that such reactions are general to many metals. Thus, as was speculated upon in the preliminary discussion of the biological chemistry of gold (Section 1.2), there is some evidence that thiol compounds influence the metabolism of gold and therefore changes in the concentrations of cysteine, glutathione and other small sulphhydryl containing molecules in plasma may, like albumin, be associated with the size of an 'active' gold pool and hence with clinical response to chrysotherapy.

Removal of aurothiomalate from the circulation and uptake into tissues would involve mechanisms of thiol association and exchange but at the same time, as has been noted above for mercury, interactions of gold with the intracellular thiol compounds, particularly metallothionein, will further influence movements of the metal. This was observed following a single dose of aurothiomalate to rats when gold was found in many tissues (Table 3.3) but particularly in the organs which have an abundance of thiol compounds - kidney and liver. Gold also accumulates in these organs when repeated administration of aurothiomalate was provided (Table 4.6) although in patients receiving chrysotherapy it was found that gold was present in all body tissues. The subcellular distribution experiments and also the studies with rheumatoid arthritis patients gave evidence of considerable relocation of gold. Intracellularly this was seen as movement from cytosol into the lysosomes. A reflection of this shift was observed on a larger scale at the region of the arthritic joint. Increased gold accumulation occurred during chrysotherapy but when treatment ceased there was a differential loss from
the synovium (i.e. from the tissue which attracts a large but transient population of lysosome-rich macrophages).

The relevance of these results to the different response observed clinically is not obvious, particularly since no data are available whereby tissue gold concentrations could be related to the outcome of treatment.

Mechanisms of action of the gold which arrives at the active site(s) also require consideration. The experiments performed to examine interrelationships between the metal and other trace-elements were of particular interest. As was noted in the first Chapter, in rheumatoid arthritis the serum concentrations of copper and zinc are increased and decreased respectively. (72) As the patient progresses towards remission as a consequence of successful treatment, the concentrations revert towards normal. Copper complexes have occasionally been used as therapeutic agents to control rheumatoid arthritis but gold compounds have proven to have greater efficacy in producing remission. These observations suggest (together with the biochemical hypothesis of Gerber - Section 1.4.3) that some aspects of rheumatoid arthritis are related to alterations in metal metabolism and that by influencing metal concentrations the course of the disease may be modified. Furthermore, gold administered therapeutically distributes to all organs and some patients receiving chrysotherapy develop symptoms of gold toxicity which involve several sites in the body; skin, bone-marrow, kidney etc. (106) Therefore if gold is effective as an anti-arthritic agent by interacting with other metals and if gold toxicity also involves metal interactions, the changes in metal concentrations in a wide range of body tissues (Fig. 4.12) following chronic administration of gold, should not be unexpected.

From the biochemical changes associated with rheumatoid arthritis and from the benefit in some patients following therapeutic administration, copper metabolism is one important area where gold may exert an effect. Further evidence that copper and gold interactions are important is derived from the signs and symptoms of gold toxicity. Many of the toxic effects of gold are similar to those observed in humans with abnormal metabolism of copper, e.g. Wilson's disease or exposed to high chronic levels of copper, e.g. workers in the plating industry. (229). Similarly, the toxic effects of gold in animals resemble closely those of copper toxicity (e.g. renal tubular damage, hepato-biliary dysfunction, deposition of metal in the eye). This is not surprising for copper and gold have similar physico-chemical properties and are in the same sub-group (1B) of the Periodic Table (Table 1.1).
The results presented in Chapter 4 (Figs. 4.9–4.12 and Tables 4.3–4.6) provide the first report of what is a dramatic and diverse effect of aurothiomalate upon the metal content of body tissues and fluids (other than serum). The changes found in the concentrations of the essential elements were similar to those which have been found in animals exposed to other metals e.g. mercury (198,199), bismuth (199) and cadmium. (199–202) In such studies it was the increase in renal copper which was the most sensitive phenomenon with an increase of 560% in the aurothiomalate treated rats (Table 4.3). This copper was shown by gel filtration to be associated with a low molecular weight protein, gold and zinc in the cytosol and by electron microprobe analysis to be coupled with copper in lysosomes.

The liver plays a fundamental role in the physiology of copper (230,231) and, as was shown in the work reported in the previous Chapters, is also important in the metabolism of gold. Copper entering hepatocytes passes to a low molecular weight protein (221) and is then incorporated into other proteins (caeruloplasmin, superoxide dismutase). Furthermore, bile is the principle excretory route for copper and hepatic lysosomes, into which the copper is known to be concentrated, discharge into the bile. Non-protein bound copper is highly toxic so that any displacement of copper from proteins might be expected to have profound effects.

A mechanism of gold toxicity could therefore be envisaged whereby; (i) gold enters cells e.g. hepatocytes and displaces copper from lysosomal metal binding proteins, (ii) free copper has a direct cytotoxic effect which would be manifested if (a) the liver already contained a large copper load or (b) there was a prior deficiency or poor ability to synthesise copper binding protein(s), (iii) displaced copper induces the formation of additional metal binding protein which spills into the circulation, is filtered at the glomerulus and is reabsorbed by renal proximal tubular cells. This protein also sequesters other metals producing deficiencies elsewhere (e.g. skin).

This mechanism then introduces other parameters into cellular reactions involving gold. The effect of this is to provide further points at which individual patient variation can be envisaged. The validity of the mechanism could be tested by investigating the details of copper and metallothionein metabolism in patients and experimental animals receiving gold.

It is recognised however that neither this nor any other single mechanism can account for all of the variation in response to gold which is encountered.
during chrysotherapy. Furthermore, factors such as the activity of the humoral or cellular immune mechanisms (232-234), which feature in the aetiology of rheumatoid arthritis, will be superimposed upon the direct effects of gold.

Conclusions

The main conclusions derived from these clinical and experimental studies with sodium aurothiomalate are:

1. Routine measurements of total gold concentrations in serum and/or urine of patients receiving chrysotherapy are of no value in predicting the response to gold. Attempts to manipulate treatment to maintain a 'therapeutic blood level' do not result in more patients showing improvement and fewer patients with gold toxicity.

2. Previous assertions that gold and its carrier molecule rapidly dissociate are not correct and some aurothiomalate remains as the intact drug. Compartmentalization of the plasma gold is achieved by albumin and by thiol compounds in the blood.

3. Gold is removed from the circulation into all tissues but particularly the kidney and liver. Further localisation of gold into tissues which contain or attract macrophages, e.g. the rheumatoid synovial membrane, also occurs. It was shown that the kidney and liver gold is associated intracellularly with a cytosolic low molecular weight protein which also contains copper and zinc but that much of this gold is subsequently relocated within lysosomes.

4. Administration of sodium aurothiomalate has a diverse effect upon trace metals at many sites within the body. Interactions with copper and metallothionein metabolism may represent a mechanism of action of gold and of gold toxicity.
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