J. Pharm. Pharmacol. 1992, 44: 467–471
Received November 6, 1991

Interaction and Aggregation of Sodium Aurothiomalate and Poly L-Lysine

ROBERT A. BROWN AND TERRI M. WELLS*

*Experimental Pathology Department, Institute of Orthopaedics, Royal National Orthopaedic Hospital, Brockley Hill, Stanmore, Middlesex HA7 4LP and *Kennedy Institute for Rheumatology, Bute Gardens, Hammersmith, London, UK*

Abstract—This report describes the interaction and aggregation of sodium aurothiomalate with polylysine, a potentially useful property, both as a model for drug-protein interaction and as a means of reversibly immobilizing sodium aurothiomalate. Sodium aurothiomalate formed stable precipitates with polylysine at neutral pH, ionic strengths below 1 M NaCl, and optimally, at sodium aurothiomalate to lysine ratios of less than one. The interaction could be demonstrated both by precipitation (which was sensitive to the size of polylysine polymer) and by using polylysine immobilized to Sepharose. Precipitation could be inhibited by addition of the organic thiomalate moiety alone. These findings indicate that the interaction of sodium aurothiomalate with polylysine is by electrostatic bonding of the thiomalate (mercaptosuccinate) moiety and the ε-amino groups of lysine residues. At suitable molar ratios this will link together many polylysine chains leading to precipitation. This represents a potentially valuable interaction for immobilization of the drug and in the formation of conjugates.

The use of gold or chrysotherapy in the treatment of rheumatoid arthritis dates back over 50 years (Forestier 1935). Despite its wide acceptance as a successful second line of treatment, its mode of action and which part of the drug is effective, remains uncertain. In the most widely used injectable form (sodium aurothiomalate) it is controversial whether the gold or the organic, thiomalate moieties are active (Freedman 1985; Taylor et al 1985). Sodium aurothiomalate comprises gold, linked through sulphur to the organic moiety of thiomalate (Puddephatt 1978; Freedman 1985). In a recent limited trial of thiomalic acid (mercaptosuccinic acid) alone, there was no indication of the therapeutic benefit which is normally associated with aurothiomalate (Rudge et al 1988) suggesting that the gold moiety at least is necessary. Sodium aurothiomalate is 98% protein-carried in serum (Lorber et al 1983), largely bound to albumin (McQueen & Dykes 1969; Danpure 1976; Smith et al 1985) which is also used as a model for its interaction with protein in general. It is suggested that protein binding is through the gold and the free thiol group on albumin, with release of the thiomalate. However, the reaction is poorly understood and probably includes contributions from other mechanisms including binding of the complete aurothiomalate molecule (Brown & Smith 1980; Taylor et al 1985). Mason (1977) has also drawn attention to the role of electrostatic bonding between carboxyl groups of the aurothiomalate and cationic groups of the protein.

Clearly, a great deal remains to be learned, not only of the mechanism of action of aurothiomalate in rheumatoid disease, but also how it interacts with plasma and tissue proteins. As part of a study aimed at the production of useful gold-protein conjugates we have investigated the direct interaction of sodium aurothiomalate with polmers of L-lysine. Polylysine is useful here as a carrier to increase the gold content of the final conjugate. We report here that this interaction produces gold-polylysine aggregates which can become insoluble under physiological conditions. This stable interaction may be useful both as a model of sodium aurothiomalate-protein interaction and as a mechanism for altering and improving the way in which this drug is administered.

Correspondence: R. A. Brown, Experimental Pathology Department, Institute of Orthopaedics, Royal National Orthopaedic Hospital, Brockley Hill, Stanmore, Middlesex HA7 4LP, UK.

Materials and Methods

Materials

Poly-L-lysine HCl, mean molecular weights 17300 Da and 3600 Da and cyanogen bromide-activated Sepharose 4B were from Sigma Chemicals (Poole, UK). Gold chloride standard solution (in 0·5 M HCl) was Spectrosol grade for atomic absorption spectroscopy, from BDH Chemicals (Poole, UK). 4-Methyl-2-pentanone (MIBK) was of spectrophotometric grade, ACS reagent, from Aldrich Chemicals (Gillingham, UK). Sodium aurothiomalate (also known as gold sodium thiomalate and myocrisin) was obtained from May and Baker (Dagenham, UK) as an injectable preparation, containing 50 mg per 0·5 mL vial. Disodium aurothio [2, 3-^{14}C]malate (sp. act. 307 μCi mmol^{-1}) was kindly donated by K.W. Clow of May and Baker Ltd. Counting was performed on a Packard Tricarb 4000 scintillation counter in Scintillator 299 emulsifier, Packard (Groningen, Netherlands). Atomic absorption spectroscopy was performed on an IL 151 instrument.

Methods

Interaction of polylysine and sodium aurothiomalate, [^{14}C]sodium aurothiomalate or gold chloride was evaluated in 50 mM Tris HCl buffer, pH 7·5 or 100 mM sodium phosphate buffer pH 7·5 at room temperature (21°C). Solutions of polylysine, typically 1 to 4 mg mL^{-1}, and sodium aurothiomalate (at 1 to 5 mg mL^{-1}, and adjusted to give appropriate ratios with the lysine) were mixed and allowed to precipitate for 10 min prior to separation of

insoluble aggregate by centrifugation (2000 *g*, 10 min). After washing in the same buffer, precipitates were redissolved in 1 mL 0·1 M NaOH for further analysis. Gold was determined by atomic absorption spectroscopy (Lorber et al 1968, 1983) relative to a sodium aurothiomalate standard. Sample preparation was as follows: sample in 1 mL was added to 1 mL of saturated potassium permanganate, 2 mL of 8 M HCl was added, mixed and heated to 100°C on a heating block for 10–20 min to decolourize. Two mL of MIBK (4-methyl-2 pentanone) was added to cooled samples, tubes were stoppered and shaken on a horizontal mixer for 2 min. The MIBK layer was removed and analysed on an IL 151 atomic absorption spectrophotometer.

Polylysine was determined by Folin–Lowry total protein assay (Lowry et al 1951) using bovine serum albumin as the standard protein solution. Standard solutions of polylysine behaved in the same way as natural proteins in this assay, giving a good calibration curve which was coincident with the albumin standard. Variations in the conditions for aggregation were by addition of NaCl (0·5–4·0 M) to the reaction buffer and by the use of 50 mM Tris solution, adjusted to pH, 1, 3, 5, 7, 9, 11 or 13 by addition of conc. HCl or NaOH. Analysis of aggregation between pH 4·55 and 7·5 and between 7·0 and 9·0 utilized 100 mM NaH_2PO_4 and Na_2HPO_4 buffers, respectively. Precipitation was monitored by solution turbidity at 450 nM (Pye SP 8/100).

Polylysine-Sepharose 4B was prepared using commercially activated CNBr Sepharose. Twenty four mg (g gel dry weight)$^{-1}$ of polylysine (17 300 Da) was allowed to react with the Sepharose under conditions recommended by the manufacturer. The gel was poured into a column, 2·5 × 1·3 cm diameter and eluted at 14 mL h^{-1} with 0·12 M NaH_2PO_4, 0·2 M NaCl pH 7·0. Sample material, 1 mg of gold, either as sodium aurothiomalate or as gold chloride was pumped through the gel and the effluent monitored at 230 nm (Uvicord flow monitor, LKB). Bound and unbound fractions were assayed for gold as above.

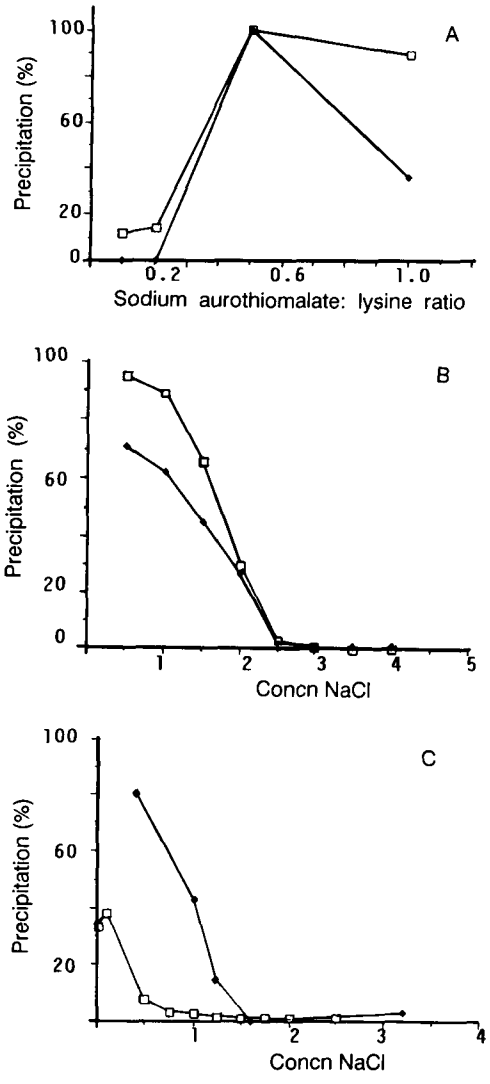

FIG. 1. A. The proportions of gold (◆) and polylysine (□) which precipitated at various sodium aurothiomalate:lysine ratios. Polylysine (17 300 Da); precipitation was in 50 mM Tris-HCl pH 7·5, room temperature (21°C) in the absence of NaCl. B. The effect of increasing concentrations of NaCl, present during precipitation, on the precipitation of gold (□) and polylysine (◆). Lysine:sodium aurothiomalate ratio was 2, pH was 7·5 (Tris-HCl) and polylysine was 17 300 Da. C. Compares the precipitation of 17 300 Da (◆) and 3600 Da (□) polylysine preparations (measured as polylysine) at increasing ionic strengths. Sodium aurothiomalate:lysine ratio was 1:1 in 10 mM sodium phosphate, pH 5·4.

Results

In the course of this investigation we have examined some aspects of the interaction of sodium aurothiomalate with polylysine. It was found that simple combination of sodium aurothiomalate and polylysine in aqueous solution (neutral pH and physiological ionic strength) produced an immediate, flocculent precipitate. The precipitate was normally white but became yellow at high levels of gold. Precipitation was dependent on the lysine:sodium aurothiomalate ratio, size of polylysine, ionic strength and pH.

The degree of precipitation is shown in Fig. 1A where the ratio of sodium aurothiomalate to lysine was varied from 0·1 to 1. A similar pattern of precipitate formation was seen whether monitored on the basis of polylysine or gold, indicating that the aggregate contained both components. Optimal precipitation of both was at a ratio of two lysine residues per molecule of sodium aurothiomalate. The divergence of the plots as measured as gold and as polylysine, is also consistent with a sodium aurothiomalate link between pairs of lysine residues.

To investigate the importance and strength of ionic interactions between sodium aurothiomalate and polylysine, the influence of NaCl on precipitation was tested (Fig. 1B). The pattern of inhibition of aggregation was again similar, when measured as polylysine or as gold in the precipitate. Aggregation was maximal at low ionic strength and decreased in a near linear fashion betwen 1·0 and 2·5 M NaCl. Pre-formed precipitates could be re-dissolved by increasing the ionic strength. NaCl, at less than 0·5 M, had no significant effect on aggregation of this size of polymer. Fig. 1C compares the formation of precipitable aggregates using two sizes of polylysine (3600 and 17 300 Da). The smaller polymer was precipitated to a lesser extent and was inhibited from aggregating at a lower ionic strength than the 17 300 Da

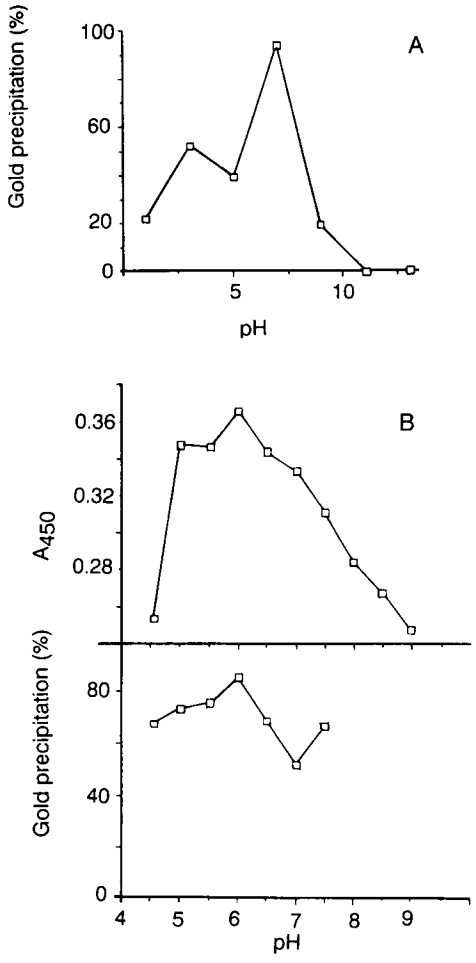

FIG. 2. Effect of pH on precipitation. A. The effect of pH on the amount of gold precipitated. A similar pattern was followed by the polylysine. Precipitation was in Tris solution (without NaCl) at a sodium aurothiomalate:lysine ratio of 0·5. B. Detailed analysis of the influence of pH on precipitation (using sodium phosphate buffered solutions) monitored as sample turbidity (upper panel) and as precipitation of gold (lower panel).

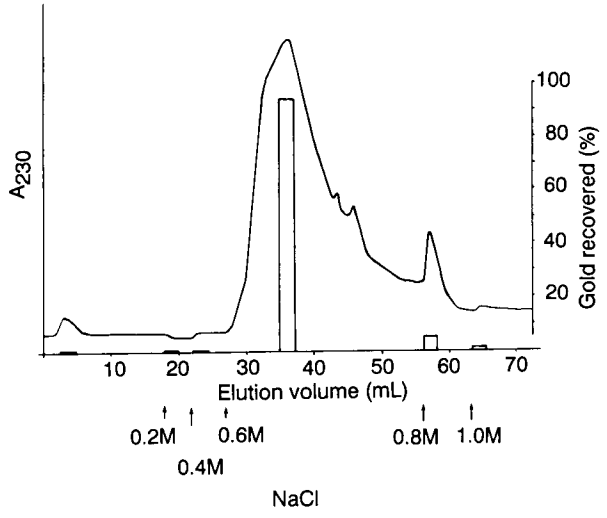

FIG. 3. The elution of sodium aurothiomalate from polylysine-Sepharose 4B (monitored by A_{230}; full scale = 0·2) using stepwise NaCl increments of 0·2, 0·4, 0·6, 0·8, 1·0 M. Fractions were subsequently pooled and analysed for gold. The columns show recovery of gold at each stage as a percentage of the total recovered gold. Recovery of gold for this experiment was 83%.

polymer. Precipitation of the 3600 Da polylysine was almost completely suppressed above 0·5 M NaCl.

Fig. 2 indicates the pH sensitivity of this aggregation as monitored by the proportion of gold which was precipitated. Over a broad pH range, in Tris-containing solutions (Fig. 2A), substantial precipitation was obtained between pH 3·0 and 7·0. Using phosphate buffers, over the range pH 4·55 to 9·0, the degree of precipitation was monitored by changes in turbidity (Fig. 2B). By this measure there was a broad optimum for precipitation around pH 6·0. A similar, though not identical, pattern was seen for the amount of gold precipitated, indicating that binding of the sodium aurothiomalate to precipitates can vary. This was supported by differences observed using different buffers at the same pH. Sodium phosphate and Tris-HCl buffers at pH 7·5 gave the same level of turbidity (within 1·2%) but the gold content of the Tris precipitate was 25% higher. As in the case of ionic strength, preformed precipitates could be redissolved by extremes of pH.

To examine the basis of this association using an alternative method, sodium aurothiomalate was allowed to interact with immobilized polylysine, using a column of polylysine-Sepharose 4B. Sodium aurothiomalate (1 mg) was applied to the column at low ionic strength and subsequently eluted in a stepwise manner with increasing concentrations of NaCl (Fig. 3). Recovery of the sodium aurothiomalate, monitored as gold, was 83%, most of which was eluted with 0·6 M NaCl. Hence, the interaction is also demonstrable by solid phase adsorption, although binding appeared weaker than in the precipitate, as judged by the lower NaCl concentration needed to cause dissociation (Fig. 1B).

To study the relative contribution of the thiomalate moiety to this interaction, gold chloride was substituted for sodium aurothiomalate. Decreasing concentrations of gold chloride were mixed with a 1 mL solution of 17300 Da polylysine (1 mg mL^{-1}), in the same way as before, to give increasing lysine: gold chloride ratios of 1, 2, 5 and 10. In no case did a precipitate form, indicating that the thiomalate is necessary for aggregation and precipitation. Similarly, the precipitation of sodium aurothiomalate (measured as gold) with polylysine was not suppressed in the presence of gold chloride (Fig. 4). However, since no precipitate was formed it remained possible that free gold was bound to the polylysine, but did not cause or inhibit precipitation. To test this, gold chloride was applied to a column of polylysine-Sepharose, at pH 7·0. All of the recovered gold (86%) was eluted in the unbound fraction from the column, indicating that little or no binding occurs. The reverse pattern of elution (i.e. complete binding) was obtained for the same amount of gold loaded as sodium aurothiomalate. In this case no unbound gold was detected, but 77% of the sample gold was recovered by elution with 1 M NaCl. The diametrically opposite behaviour of gold chloride and sodium aurothiomalate is consistent with the suggestion that sodium aurothiomalate binds via its organic moiety.

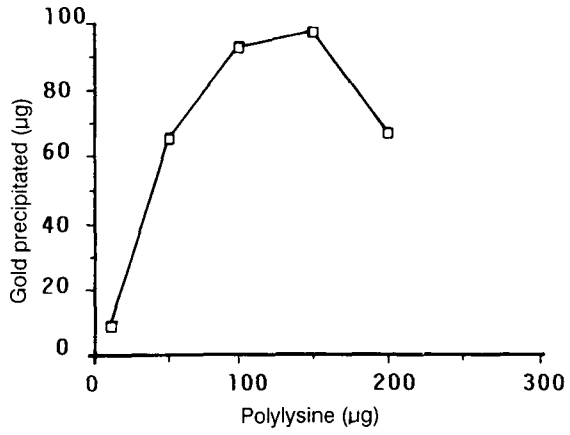

FIG. 4. Failure of gold chloride to inhibit sodium aurothiomalate:polylysine precipitation. Gold chloride standard solution, diluted and buffered to pH 7·5 with 100 mM sodium phosphate (containing 100 μg of gold) was mixed with polylysine (17 300 Da) solutions containing 10–200 μg. In no case did a precipitate form. Sodium aurothiomalate (100 μg) was then added and precipitates were spun down and redissolved, prior to analysis for gold. The effect of polylysine concentration on this gold precipitation is shown (sodium aurothiomalate:lysine range was 3·6–0·18).

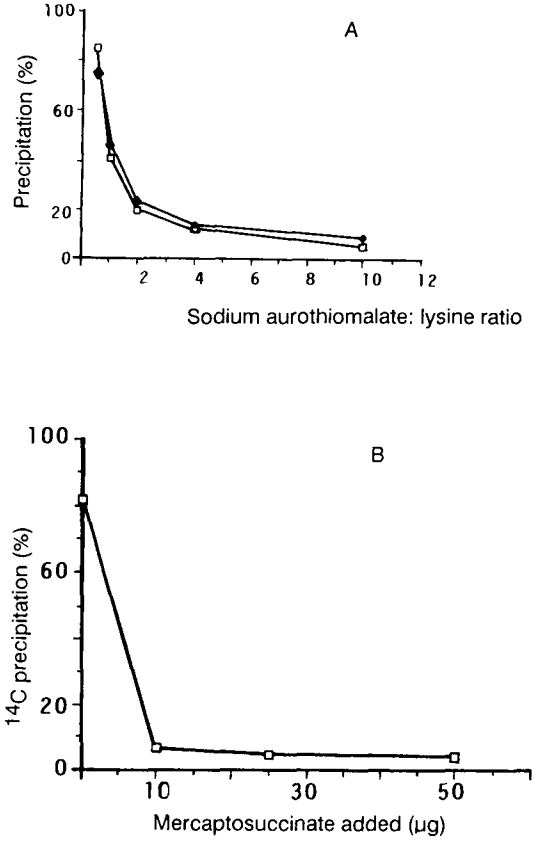

FIG. 5. A. Effect of sodium aurothiomalate:lysine ratio on precipitation of gold and [^{14}C]sodium aurothiomalate. [^{14}C]Sodium aurothiomalate was mixed with various amounts of 17 300 Da polylysine (pH 7·5, 100 mM sodium phosphate). The resulting precipitates were washed, redissolved in 0·1 M NaOH and counted. Levels of [^{14}C]sodium aurothiomalate are expressed (□) relative to total recovered d min^{-1} (recovery of ^{14}C ranged from 92 to 110%: mean = 104%). Each point is the mean of two samples which, for all points, differed by less than 2%. Gold levels (◆) were also determined on the pelleted material and expressed relative to the total gold added to each test. Each point represents the mean of duplicate measurements. B. Effect of mercaptosuccinic acid on precipitation of [^{14}C]sodium aurothiomalate. Constant amounts of [^{14}C]sodium aurothiomalate and polylysine (17 300 Da) were mixed in a molar ratio of 0·2:1 (12·5 μg sodium aurothiomalate per test). To this was added increasing amounts of mercaptosuccinic acid (to give molar ratios of mercaptosuccinate:sodium aurothiomalate between 0 and 10·7). The pH was maintained by addition of 100 mM sodium phosphate buffer, pH 7·5 without NaCl. Each point is the mean of duplicate determination; standard errors were within the symbol.

Further evidence for this mode of interaction was obtained using [^{14}C]sodium aurothiomalate. The ^{14}C-labelled drug was precipitated with polylysine 17 300 Da as before, washed in buffer and redissolved prior to counting and gold analysis. At a sodium aurothiomalate:lysine ratio of 0·6, 82% of the radioactivity and 91% of the gold precipitated. Only 14% of radioactivity was precipitated at a ratio of 3. Recovery of labelled drug was greater than 95% in each case. Fig. 5A illustrates this precipitation as both [^{14}C]sodium aurothiomalate and gold. The similarity in precipitation of gold and total radioactivity again indicates that the sodium aurothiomalate remains as an intact unit, under these conditions, during binding and aggregation. It seems improbable, then, that the gold dissociates and is responsible on its own for aggregation.

Involvement of the organic moiety of the drug (i.e. mercaptosuccinic acid) was further tested by adding increasing amounts to a precipitating mixture of [^{14}C]sodium aurothiomalate and polylysine (as in Fig. 5A), to test for competitive inhibition. At a molar ratio of 0·5 (sodium aurothiomalate:lysine) ^{14}C precipitation was reduced to less than one tenth by addition of mercaptosuccinate (Fig. 5b). Maximal inhibition was produced at a mercaptosuccinate: sodium aurothiomalate molar ratio of 2. Interestingly, mercaptosuccinate alone was unable to precipitate polylysine over a range of molar ratios (mercaptosuccinic acid:lysine 0·5–5). The reason for this effect is unknown but may be related to a difference in solubility resulting from the substitution of the organic thiomalate moiety with gold.

Discussion

This study forms part of a programme to develop effective conjugates of sodium aurothiomalate and biologically active proteins (Brown 1988; Ejim et al 1991). Clearly, poly L-lysine represents a promising carrier for this drug, having a high capacity and good stability. Although precipitation was used extensively in this report, it is clear that soluble complexes form equally readily. The nature of this interaction under a variety of conditions suggests that binding is chiefly electrostatic. The peak ratio for precipitation (though not necessarily for binding) of two lysine residues per sodium aurothiomalate suggests the possibility that the ε-amino group of the polylysines can interact with each of the two carboxyl groups on a sodium aurothiomalate molecule.

The finding that both gold-polylysine and [^{14}C]sodium aurothiomalate-polylysine precipitate in a similar pattern, demonstrates that binding does occur without dissociation of the gold, in contrast to sodium aurothiomalate-albumin binding in-vivo (Brown & Smith 1980). However, free

sulphydryl groups, available in-vivo, may favour this form of dissociation. Failure of gold chloride to bind or aggregate polylysine, or to inhibit polylysine sodium aurothiomalate precipitation (in contrast to mercaptosuccinate) indicates that the gold moiety plays little part in this interaction. It is proposed that interaction occurs through electrostatic binding of thiomalate and lysine residues, leading to cross-linking, aggregation and ultimately precipitation of polylysine chains. The importance of cross-linking and polymer size was indicated by the finding that the smaller polymer of lysine was less precipitable. Some degree of polymerization through the gold moiety cannot be ruled out (particularly since mercaptosuccinic acid did not precipitate polylysine) and this, in turn, could be sensitive to ionic strength (Grootveld & Sadler 1983). The effect of pH on sodium aurothiomalate-polylysine binding and precipitation was complex since the results suggested that precipitates with different gold contents were formed.

Stability of sodium aurothiomalate-polylysine at physiological pH and ionic strength suggests that it could be useful in the formation of drug delivery conjugates. For example, intramuscular depots of aggregated sodium aurothiomalate-polylysine could be formed by injection of the two components to the same site. The polylysine is easily degraded by tissue proteases which would release soluble sodium aurothiomalate-lysine (Ryser & Shen 1978). Conjugation to polylysine would in itself alter the pharmacodynamics of sodium aurothiomalate since binding to tissue proteins (which presumably occurs normally, following intramuscular injection) would be blocked. Previous work, on methotrexate substitution of poly L-lysine, has indicated that cellular uptake of the drug is improved (Ryser & Shen 1978; Shen & Ryser 1978). Both of these effects could prove to be pharmacologically desirable, but this would require tests in-vivo, since there is no agreed mechanism by which sodium aurothiomalate is effective.

An improved understanding of this reaction may have useful applications outside the field of rheumatology: for example as a labelled probe to mark exposed cationic sites on the surface of proteins, both biochemically and in the electron microscope. A similar principle has been tried using polylysine-coated colloidal gold particles (Skutelsky & Roth 1986). However, it is in the investigation of sodium aurothiomalate distribution in-vivo and its action in rheumatoid arthritis where this simple model of protein binding will be most valuable. The apparent electrostatic nature of binding through the thiomalate contrasts with the emphasis placed by many workers on gold mediated protein binding. It may be that the latter form of binding occurs after the thiomalate-mediated interaction with tissue proteins, as the gold moiety is dissociated. It seems likely, given the strength of its binding to this common, basic amino acid, that such interactions with tissue proteins at the injection site will influence its subsequent distribution. In addition, the production of a re-dissolvable precipitate is a considerable advantage for analysis, as it allows rapid separation of bound from free sodium aurothiomalate without recourse to other separation techniques.

Acknowledgements

We are grateful to Mrs Elisabeth Cooke for technical assistance, Dr M. J. Harvey for his helpful comments, to May and Baker for a gift of [^{14}C]sodium aurothiomalate and to the Central Blood Laboratories Authority for their financial support.

References

Brown, R. A. (1988) Pharmaceutically active conjugates having improved body tissue binding specificity. Patent application number PCT/GB87/00854

Brown, D. A., Smith, W. E. (1980) The chemistry of the gold drugs in the treatment of rheumatoid arthritis. Chem. Soc. Rev. 9: 217–240

Danpure, C. J. (1976) Interaction of aurothiomalate with human serum albumin in vitro. Biochem. Soc. Trans. 4: 161–163

Ejim, O. S., Wells, T., Harvey, M. J., More, J., Brown, R. A. (1991) Directed gold therapy using myocrisin coupled to fibronectin fragments. Br. J. Rheumatol. 30 (Suppl. 1): 19

Forestier, J. (1935) Rheumatoid arthritis and its treatment by gold salts. The results of 6 years' experience. J. Lab. Clin. Med. 20: 827–840

Freedman, P.S. (1985) Myocrisin: review of pharmacology. In: Nuki, G., Gumpel, J. M. (eds) Myocrisin, 50 Years' Experience MCI, London, pp 25–30

Grootveld, M. C., Sadler, P. J. (1983) Differences between the structure of the anti-arthritic gold drug "Myocrisin" in the solid state and in solution: a kinetic study of dissolution. J. Inorg. Biochem. 19: 51–64

Lorber, A., Cohen, L., Chang, C. C., Anderson, H. E. (1968) Gold determination in biological fluids by atomic absorption spectrophometry: application to chrysotherapy in rheumatoid arthritis patients. Arth. Rheum. 11: 170–177

Lorber, A., Vibert, G. J., Harralson, A. F., Simon, T. M. (1983) Unbound serum gold: procedure for quantitation. J. Rheumatol. 10: 563–567

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275

Mason, R. W. (1977) The binding of aurothiomalate to plasma proteins in vitro. Pharmacology 15: 536–544

McQueen, E. G., Dykes, P. W. (1969) Transport of gold in the body. Ann. Rheum. Dis. 28: 437–442

Puddephatt, R. J. (1978) The Chemistry of Gold. Elsevier Scientific, Amsterdam, pp 247–252

Rudge, S. R., Perrett, D., Kelly, M. (1988) Failure of oral thiomalate to act as an alternative to intramuscular gold in rheumatoid arthritis. Ann. Rheum. Dis. 47: 224–227

Ryser, H. J. P., Shen, W.-C. (1978) Conjugation of methotrexate to poly (L-lysine) increases drug transport and overcomes drug resistance in cultured cells. Proc. Natl. Acad. Sci. USA 75: 3867–3870

Shen, W. C., Ryser, H. J. P. (1978) Conjugation of poly-L-lysine to albumin and horseradish peroxidase: a novel method of enhancing the cellular uptake of proteins. Ibid. 75: 1872–1876

Skutelsky, E., Roth, J. (1986) Cationic colloidal gold—a new probe for the detection of anionic cell surface sites by electron microscopy. J. Histochem. Cytochem. 34: 693–696

Smith, W. E., Brown, D. H., Cappell, H. A. (1985) Gold binding sites in the plasma of patients with rheumatoid arthritis undergoing treatment with gold sodium thiomalate. Inorg. Chim. Acta 106: 423–425

Taylor, A., King, L. J., Marks, V. (1985) Tissue deposition and excretion of gold and ^{14}C in rats treated with sodium aurothio [1,4-^{14}C] malate or thio [1,4-^{14}C] malic acid. Xenobiotica 15: 221–226