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INHIBITION OF TRYPSIN AND PAPAIN BY SODIUM AUROTHIOMALATE MEDIATED BY EXCHANGE REACTIONS

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1 Sodium aurothiomalate has been shown to participate in exchange reactions leading to the inhibition of trypsin; for this exchange to take place it was necessary to include in the test system a suitable thiol, such as N-acetyl-cysteine.

2 Neither N-acetyl-cysteine nor aurothiomalate on their own had any inhibitory action on trypsin.

3 The results indicate that aurothiomalate dissociates in the presence of a carrier to form thiosuccinate and gold.

4 The gold is responsible for trypsin inhibition since independent experiments demonstrated that the total concentration of thiosuccinate was insufficient to cause the observed inhibition of trypsin.

5 Bovine serum albumin was shown to act as a carrier in place of N-acetyl-cysteine.

6 It is known that histidine in the active centre of trypsin binds heavy metal ions with consequent inhibition of the enzyme. In this study, imidazole was shown to act as a carrier for gold from aurothiomalate to trypsin resulting in inhibition. This inhibition by gold was reversed when higher concentrations of imidazole were added to the test system due to competition for the trypsin-bound gold by imidazole.

7 Conversely, the thiol enzyme papain was re-activated in the presence of low concentrations of sodium aurothiomalate and inhibited by higher concentrations of this reagent in a biphasic manner. This observation will be discussed in relation to the dissociation of sodium aurothiomalate.

8 These observations can also be explained in terms of exchange reactions involving thiols and free metal ions.

Introduction

Sodium aurothiomalate has been extensively used as a drug (Myocrisin, M & B) in the treatment (Gottlieb, 1979) of rheumatoid arthritis, an inflammatory disorder of connective tissues accompanied by increased proteolytic destruction of cartilage (Vernon-Roberts, 1979). The mechanism by which sodium aurothiomalate reduces proteolysis is unclear. The results described below demonstrate an indirect role for sodium aurothiomalate in the inhibition of two proteolytic enzymes. We have employed the proteolytic enzyme, trypsin, which has three well established expressions of enzymatic activity, viz. proteolytic, esterase and β-naphthylamidase activity. On its own, sodium aurothiomalate has no inhibitory activity against any of these three activities of trypsin. When N-acetyl-cysteine or bovine serum albumin was included in the buffer system to facilitate the exchange of gold, the activity of trypsin was inhibited by incremental additions of sodium aurothiomalate.

In the present study the concentration of sodium aurothiomalate participating in exchange reactions leading to effective inhibition of trypsin was far lower than the corresponding concentration of thiosuccinate required to produce an equivalent inhibition of trypsin by thiol-disulphide exchange (Steven & Podrazký, 1978). It is known that metal ions (e.g. Ag^+) (Martinek, Savin & Berezin, 1971) bind to a histidine residue in the active centre of trypsin, causing inhibition. It is also known that metal ions (e.g. Ag^+, Cu^{2+}, Hg^{2+}) participate in reactions involving thiols and disulphides leading to biphasic re-activation and subsequent inhibition of latent trypsin; a detailed account of these reactions has already been published (Steven, Podrazký, Al-Habib & Griffin, 1979). The evidence presented below indicates that sodium aurothiomalate, in the presence of a suitable carrier, can dissociate in such a manner that the gold is transferred to the active site of trypsin resulting in the inhibition of all three enzymatic activities of trypsin.

The action of sodium aurothiomalate on a thiol enzyme, papain (which requires to have its thiol group in the reduced and unsubstituted form for

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enzymatic activity to be expressed) has also been studied. The experimental results obtained were readily explained on the basis of similar exchange reactions taking place.

Methods

Assay of enzymatic activity

Proteolytic activity Two methods were used: (a) the solubilization of fluorescein-labelled peptides from fluorescein-labelled insoluble polymeric collagen fibrils (Steven *et al.*, 1975; Steven & Podrazký, 1978) and (b) the cleavage of fluorescein-labelled peptides from fluorescein-labelled casein. In the latter assay the enzymatic digestion of 12 mg substrate per tube was stopped by adding trichloroacetic acid to the reaction mixture to give a final concentration of 5% w/v trichloroacetic acid. The soluble peptides (100 μl sample from each tube) were mixed with 2.0 ml 2% w/v $NaHCO_3$ and assayed fluorimetrically (Steven & Podrazký, 1978). In each analysis, the residual proteolytic activity was expressed as a percentage of the control trypsin activity to which no inhibitors were added. Product formation was linear over a 2 h period with 2 μg of added trypsin in both these assays.

β-Naphthylamidase activity The fluorimetric assay procedure of MacDonald, Ellis & Reilly (1966) was employed with a 1 h incubation period during which time the product formed in the presence of 2 μg of trypsin was linear with time. It was observed that the addition of sodium aurothiomalate to the α-*N*-benzoyl-DL-arginine-β-naphthylamide HCl (BANA) solution resulted in precipitation; this could be overcome by adding 5 mM N-acetyl-cysteine, or 100 mM guanidinium chloride or 2 mg bovine serum albumin to the buffer system prior to adding the BANA. None of these additions caused quenching of the fluorescence exhibited by the product of enzyme digestion, β-naphthylamine. The guanidinium chloride diminished the total product formed by trypsin digestion by some 40%. This difficulty was overcome by including this agent in all the tubes of an experiment so that the initial activity of the trypsin was equivalent in all tubes in these experiments prior to adding sodium aurothiomalate.

Esterase activity The esterase activity of trypsin was assayed with N-carbobenzoxy-L-tyrosine-nitrophenyl ester (CTN) according to the method of Martin, Golubow & Axelrod (1959) over a period of 6 min. The production of nitrophenol was linear over this period for 1 μg of trypsin. Two types of esterase assay were used.

(a) *Assay with a single substrate* It was observed that bovine serum albumin alone and also N-acetyl-cysteine alone caused some cleavage of the substrate. These problems were overcome by using 30 μg of trypsin in each tube with a total volume of 3.0 ml buffer containing appropriate additions of either bovine serum albumin or N-acetyl-cysteine plus incremental additions of sodium aurothiomalate. After a suitable period, e.g. 20 min at 37°C, 100 μl aliquots were removed by microsyringe from each tube and assayed for esterase activity (Martin *et al.*, 1959). In this way, the formation of products caused by the presence of the bovine serum albumin or N-acetyl-cysteine was reduced in the final assay to a very small background level similar in magnitude to the blank value for the test system. The results obtained in these inhibition experiments with sodium aurothiomalate are presented as percentages of the control values in which the potential inhibitor was omitted.

Sodium aurothiomalate alone caused no cleavage of the substrate in 6 min, thus the direct action of sodium aurothiomalate on 1 μg of trypsin was assayed with CTN without the need for the dilution procedure described above.

(b) *Assay with two substrates simultaneously* The dilution procedure for esterase analysis offered the advantage that two substrates could be used simultaneously to assay trypsin in the presence of a potential inhibitor. In these experiments we used 30 μg of trypsin in each tube plus 12 mg fluorescein-labelled casein. We added either 2 mg of bovine serum albumin or N-acetyl-cysteine to give a final concentration of 5 mM in a total volume of 3 ml with pH adjusted to 8.0. We finally added incremental additions of sodium aurothiomalate and incubated the tubes for 1 h at 37°C. After this period of incubation, 100 μl samples were removed from each tube by microsyringe and assayed immediately for esterase activity (Martin *et al.*, 1959). The residual 2.9 ml of reaction mixture was mixed with 0.5 ml 25% w/v trichloroacetic acid to terminate the reaction and the soluble fluorescein-labelled peptides determined (Steven & Podrazký, 1978) in order to assay the caseinolytic activity of the trypsin in the presence of the potential inhibitors.

When the esterase and caseinolytic activity were simultaneously determined to obtain inhibition data in the presence of incremental additions of sodium aurothiomalate, the results were very similar to those obtained when each substrate was employed independently.

Materials

Crystalline trypsin (EC 3.4.21.4) and N-acetyl-cysteine were obtained from Boehringer, Mannheim,

Germany. Twice crystallized papain (EC 3.4.22.2), N-carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester (CTN), α-*N*-benzoyl-DL-arginine-β-naphthylamide HCl (BANA), thiosuccinic acid and bovine serum albumin were purchased from Sigma, St Louis, U.S.A. Guanidinium chloride and imidazole were purchased from BDH, Poole, and 'Myocrisin', a 2% w/v solution of sodium aurothiomalate was purchased from May and Baker, Dagenham. Fluorescein-labelled polymeric collagen fibrils were prepared as described (Steven, Torre-Blanco & Hunter, 1975), fluorescein labelling of casein was carried out in a similar manner with repeated precipitation from solution of the product by 5% trichloroacetic acid to ensure the removal of trichloroacetic acid-soluble peptides.

Results and Discussion

Sodium aurothiomalate

The proteolytic and esterase activities of trypsin were not inhibited by addition of sodium aurothiomalate alone in the concentration range 0–2 mM. Similarly, sodium aurothiomalate (0–2 mM) had no inhibitory action on the β-naphthylamidase activity of trypsin assayed with 0.1 M guanidinium chloride included in the buffer system in order to prevent precipitation (see above).

Sodium thiosuccinate

Concentrations of 0–5 mM thiosuccinate had no inhibitory action on trypsin esterase, β-naphthylamidase and caseinolytic activity although inhibition of trypsin cleavage of the fluorescein-labelled peptides from fluorescein-labelled polymeric collagen fibrils was observed (Figure 1).

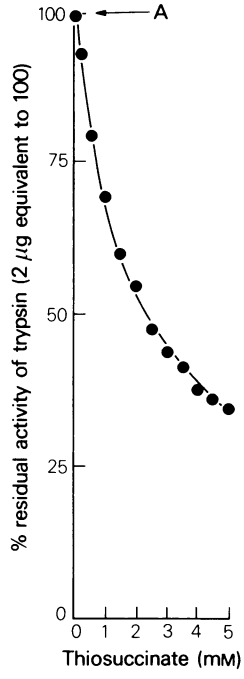

Figure 1 Thiosuccinate inhibition of trypsin assayed with fluorescein-labelled polymeric collagen fibrils: the assay was carried out for 2 h at 37°C. The arrow at A indicates the control trypsin activity (2 μg) without addition of thiosuccinate.

Sodium aurothiomalate plus bovine serum albumin

When 2 mg serum albumin/3 ml buffer was included in the assay systems, incremental additions of sodium aurothiomalate (0–1.5 mM) caused marked inhibition of trypsin: (i) β-naphthylamidase, (ii) esterase, (iii) caseinolytic activity and (iv) ability to cleave fluorescein-labelled peptides from fluorescein-labelled polymeric collagen fibrils (Figure 2). The inhibitory effect of sodium aurothiomalate was most apparent when protein substrates (iii and iv) were employed rather than synthetic, low molecular weight, substrates (i and ii). The role of the added serum albumin is to provide a mechanism for dissociation of the gold from the sodium aurothiomalate, see discussion below and references (Mascarenhas, Granda & Freyberg, 1972; Jellum, Munthe, Guldal & Aaseth, 1980).

Sodium aurothiomalate plus N-acetyl-cysteine

When 15 mM N-acetyl-cysteine was included in the buffers, this reagent had no inhibitory action on trypsin assayed with BANA or fluorescein-labelled polymeric collagen fibrils. Incremental additions of sodium aurothiomalate to the trypsin in the presence of the N-acetyl-cysteine (5 or 13 mM) resulted in marked inhibition of (i) β-naphthyl-amidase activity and (ii) cleavage of fluorescein-labelled peptides from fluorescein-labelled polymeric collagen fibrils (Figure 3). It should be noted that the highest concentration of sodium aurothiomalate employed (Figure 3ii) was approximately one tenth the concentration of sodium thiosuccinate (Figure 1) required to produce a similar inhibition using this substrate.

Clearly the inhibition shown in Figure 3(ii) could not have been due to the formation of thiosuccinate from thiomalate by the exchange of the gold initially present in the drug. The evidence would suggest that the observed inhibition is brought about by the gold released in this dissociation. This suggestion is supported by the observation that the trypsin β-naphthylamidase and esterase activity was not inhibited by either sodium aurothiomalate alone, N-

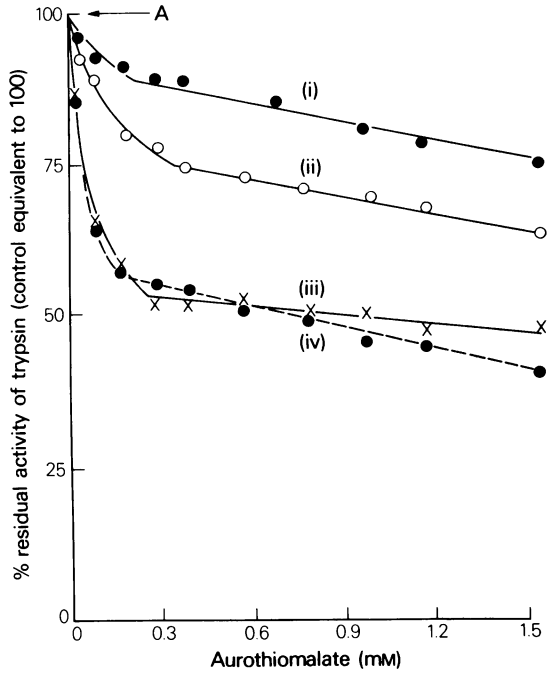

Figure 2 Sodium aurothiomalate inhibition of trypsin in the presence of bovine serum albumin. All incubations were carried out at 37°C. Curve (i) β-naphthylamidase activity after 1 h; curve (ii) esterase activity after 6 min; curve (iii) caseinolytic activity determined after 2 h on fluorescein-labelled casein; curve (iv) proteolytic activity determined after 2 h on fluorescein-labelled polymeric collagen fibrils. The arrow at A indicates the control trypsin activity in each case without addition of sodium aurothiomalate. In the absence of bovine serum albumin as carrier, 1.5 mM sodium aurothiomalate caused no inhibition.

acetyl-cysteine alone or by thiosuccinate (as stated above). In this series of experiments, the N-acetyl-cysteine plays a role in dissociating the gold from the aurothiomalate and transferring the gold to the enzyme. It is possible that cysteine residues in bovine serum albumin are the functional groups which enable this protein to participate in a similar exchange described above in Figure 2. It is not possible to study the direct action of monovalent gold, since these salts remain soluble only in the presence of excess HCl and the latter conditions are unfavourable for trypsin activity.

Sodium aurothiomalate plus imidazole

The requirement of a carrier molecule to promote the exchange of gold from sodium aurothiomalate, prior to the inhibition of trypsin taking place, has been demonstrated above. It is necessary to define whether the gold or thiosuccinate causes the inhibition of trypsin. The kinetic evidence above excludes the role of thiosuccinate and implies that gold is the effective agent, similar to the effect of silver ions binding to the histidine in the active centre of trypsin (Martinek *et al.*, 1971). We therefore employed imidazole as a carrier for gold, supplied as aurothiomalate to inhibit trypsin digestion of fluorescein-labelled polymeric collagen fibrils. In our experiments we initially had 2 μg trypsin in each tube; in the absence of added imidazole these control tubes had trypsin activity equivalent to 100% (point A on Figure 4). Incubation of trypsin with sodium aurothiomalate (100 μM) in the absence of added imidazole caused no loss in activity; this emphasizes the need for a carrier to transfer the gold from the aurothiomalate to the active centre of the trypsin molecule (see below). Similarly, addition of imidazole alone to trypsin was shown (in independent

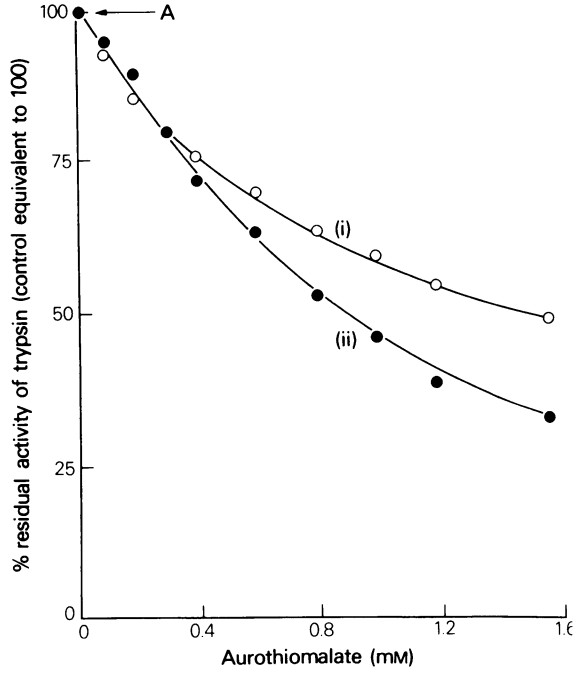

Figure 3 Sodium aurothiomalate inhibition of trypsin in the presence of N-acetyl-cysteine. All incubations were carried out at 37°C. Curve (i) β-naphthylamidase assayed with BANA in the presence of 13.8 mM N-acetyl-cysteine for 1 h; curve (ii) proteolytic activity assayed with fluorescein-labelled polymeric collagen fibrils in the presence of 5 mM N-acetyl-cysteine for 2 h. The arrow at A indicates the control activity in each case without addition of sodium aurothiomalate. In the absence of the carrier, N-acetyl-cysteine, 1.6 mM sodium aurothiomalate had no inhibitory action on the β-naphthyl-amidase activity or on the proteolytic activity of trypsin in these assay systems.

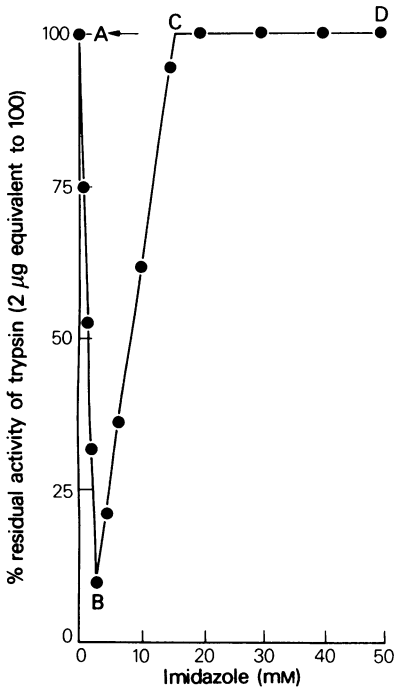

Figure 4 Biphasic inhibition and reactivation of trypsin by a fixed quantity of aurothiomalate in the presence of incremental additions of imidazole. Trypsin proteolytic activity was assayed with fluorescein-labelled polymeric collagen fibrils as substrate at 37°C for 2 h. The arrow at A indicates (i) the control digest in which no sodium aurothiomalate or imidazole was added, (ii) those tubes which contained only sodium aurothiomalate (100 μM) exhibited no inhibition of trypsin and these controls are also indicated by the arrow. Incremental additions of imidazole resulted in an initial inhibition of trypsin activity (line AB) followed by reactivation (BCD) at higher concentrations of imidazole. Imidazole (0–50 mM) alone had no action on the trypsin digestion of fluorescein-labelled polymeric collagen fibrils in marked contrast to the addition of imidazole (0–50 mM) plus aurothiomalate (100 μM), plot ABCD.

experiments) to cause no trypsin inhibition. The addition of 0–2.5 mM imidazole (line AB, Figure 4) caused marked inhibition of the trypsin in the presence of a constant amount (100 μM) of sodium aurothiomalate. Over this range, 90% of the trypsin activity was destroyed under conditions where 100 μM thiosuccinate has very little inhibitory action on trypsin digestion of fluorescein-labelled polymeric collagen fibrils (Figure 1). So it was clear that if all the sodium aurothiomalate dissociated to sodium thiosuccinate, thiol inhibition of trypsin could not account for the results presented in Figure 4, line AB. The results of Figure 4 suggest that the gold is transferred to the trypsin-active centre by the carrier molecule, in this case imidazole; subsequently the gold binds to the histidine of the active centre in the manner described for silver (Martinek *et al.*, 1971). We initially chose imidazole as a potential carrier of metals based on the published affinity of histidine in trypsin for binding silver and other metals (Martinek *et al.*, 1971; Steven *et al.*, 1979) which resulted in metal inactivation of this enzyme.

We believed that if a histidine in the active centre of trypsin bound gold with consequent inhibition (Figure 4, line AB), this inhibition might be reversed if the concentration of imidazole was increased, so as to act as a competitive binding agent for the limited supply of gold available in the 100 μM sodium aurothiomalate in the test system. The experimental results presented in Figure 4, lines BC and CD, demonstrate the regain of trypsin activity in the test system when the imidazole concentration was increased above 2.5 mM. It should be clearly understood that all the tubes used to obtain the data of Figure 4 contained a constant amount of both sodium aurothiomalate and trypsin, the only variable being the incremental addition of imidazole. We would also point out that it is not possible to employ casein or fluorescein-labelled casein followed by trichloroacetic acid precipitation in the presence of incremental additions of imidazole. Imidazole interferes with the precipitation step resulting in the total solubilization of both degraded and undegraded casein.

The mechanisms proposed for the results presented in Figure 4 are set out in Figure 5, in which reaction (a) represents the line AB of Figure 4, i.e. the transfer of gold from the drug sodium aurothiomalate to the carrier (imidazole) which then transfers the gold by exchange to the histidine in the active centre of trypsin leading to metal inhibition. The latent trypsin (metal inhibited) can be reactivated by excess imidazole (reaction (b) in Figure 5) to regain proteolytic activity (Figure 4, line BC) when all the trypsin-bound gold is exchanged with imidazole in the surrounding buffer. We believe the above series of exchange reactions involving a thiol, imidazole and histidine in the active centre of trypsin (Figures 4, 5) again illustrate the indirect role by which sodium aurothiomalate may control the activities of the enzyme trypsin. These studies with imidazole therefore complement the exchange reactions previously discussed in which N-acetyl-cysteine and bovine serum albumin were employed as carriers of gold.

It is not possible to employ monovalent gold salts at pH 8 in order to check these proposed mechanisms directly. It is probable that the proposed exchange reactions provide very low concentrations of gold at the active centre of trypsin without the precipitation of colloidal gold particles, thus enabling the control of enzymatic activity by aurothiomalate in an indirect manner.

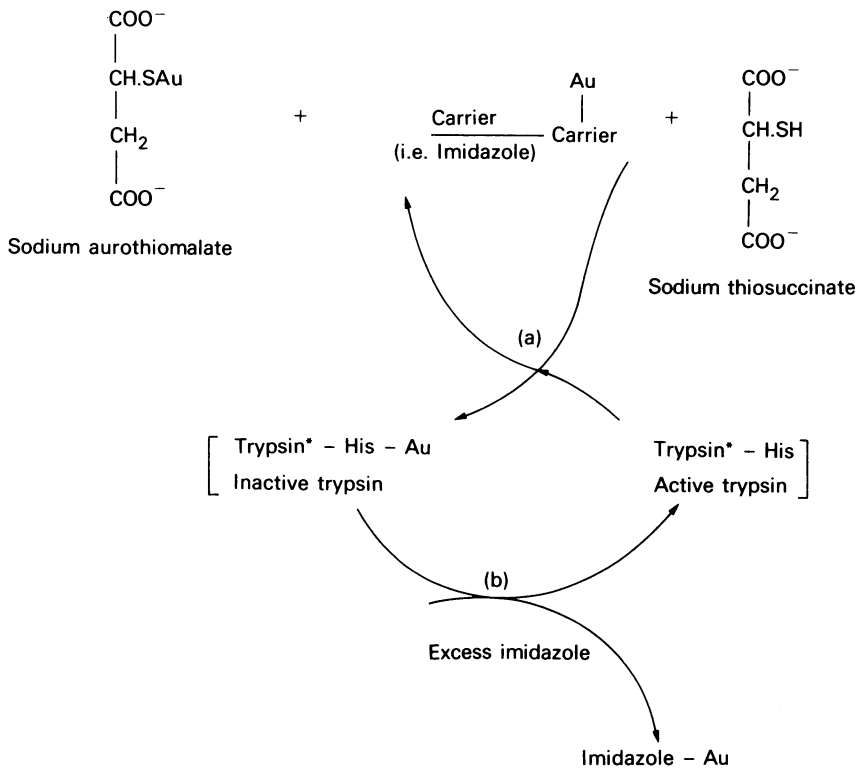

Figure 5 Proposed mechanism for the exchange of gold from sodium aurothiomalate to the active centre of trypsin mediated by imidazole.

Action of sodium aurothiomalate on papain

Enzymes which depend for their action on a thiol in their active centre, such as papain or lysosomal cathepsins, might be expected to accept gold directly from sodium aurothiomalate without the need of an intermediate carrier molecule. We employed crystalline papain to demonstrate the validity of the above hypothesis. The caseinolytic activity of commercially available papain is rather limited unless the papain is first activated by the addition of a thiol agent. We therefore used 140 μg of papain in each tube with incremental additions of sodium aurothiomalate (Figure 6). It can be seen that the thiol enzyme papain exhibited an exact reversal of the trypsin plot shown in Figure 4. In Figure 6 the line AB represents re-activation of papain due to thiol addition resulting from the dissociation of gold from sodium aurothiomalate. However, as the gold concentration is increased, inhibition of papain occurs probably due to binding of gold to the thiol in the active centre of the enzyme (Figure 6, line BC).

We conclude from the above kinetic data that aurothiomalate readily dissociates in the presence of a suitable carrier for gold and that the released gold can subsequently bind to histidine in trypsin's active centre or to cysteine in papain's active centre with the consequent inhibition of both these enzymes. The mechanisms described above involve exchanges of gold from thiol complexes and formation of histidine, imidazole or thiol complexes. Both processes lead to the reversible control of enzymatic activity (Figures 4, 6).

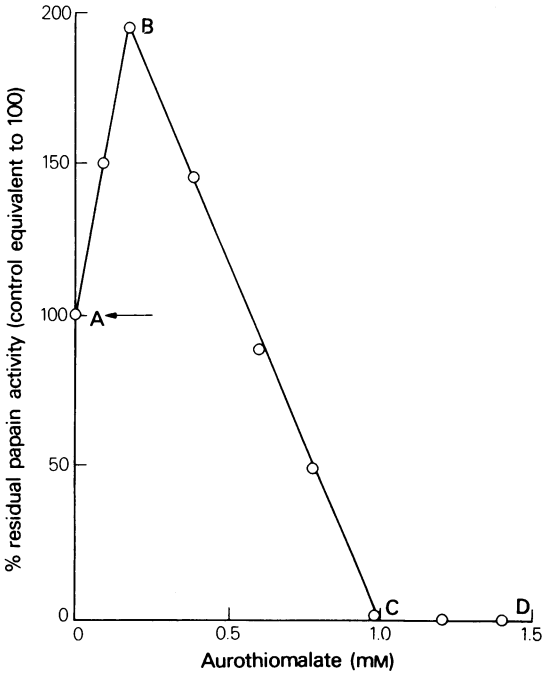

Figure 6 Biphasic activation and subsequent inhibition of papain in the presence of incremental additions of aurothiomalate. The proteolytic activity of papain was assayed with fluorescein-labelled casein as substrate in the presence of aurothiomalate for 2 h. The control tubes containing papain and no aurothiomalate are represented by the arrow at A. Increasing aurothiomalate concentrations caused an initial activation of papain (AB) followed by a subsequent inhibition (BCD) at higher concentrations. Aurothiomalate (1.5 mM) alone had no action on papain assayed under these conditions.

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