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INHIBITION OF THE DENATURATION OF HUMAN GAMMA GLOBULIN BY A MIXTURE OF D-PENICILLAMINE DISULFIDE AND COPPER

A POSSIBLE MECHANISM OF ACTION OF D-PENICILLAMINE IN RHEUMATOID ARTHRITIS

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Abstract—Mixtures of D-penicillamine disulfide and copper(II) mimicked sulfhydryl-blocking reagents (viz. gold thiomalate, *N*-ethylmaeimide and *p*-chloromercuribenzoic acid) in inhibiting the formation of acetic acid-insoluble heat-denatured human gamma globulin and hyperviscous heat-denatured bovine serum albumin and diluted human serum. These studies suggest a mechanism by which D-penicillamine, after oxidation to D-penicillamine disulfide, could inhibit the denaturation of synovial fluid gamma globulin and in this way possibly suppress rheumatoid arthritis.

D-Penicillamine disulfide, also known as oxidized D-penicillamine and 3,3,3′,3′-tetramethyl-D-cystine, is a major metabolite of D-penicillamine [1–10]. The present study demonstrates an inhibitory effect of mixtures of D-penicillamine disulfide and copper on sulfhydryl-dependent denaturation of human gamma globulin, bovine serum albumin, and diluted human serum. This inhibition may explain the beneficial effect [11] of D-penicillamine in rheumatoid arthritis.

The methods used to demonstrate inhibition of protein denaturation by mixtures of D-penicillamine disulfide and copper(II) are those that have been used to demonstrate similar inhibition by the physiologically occurring L-histidine–L-cystine–copper(II) complex [12].

MATERIALS AND METHODS

Human gamma globulin (Cohn Fraction II) was provided by the American Red Cross National Fractionation Center, Bethesda, MD; the American Red Cross was supported in part by NIH grant HE 13881 HEM. Prior to use, as previously described [13], gamma globulin solutions were treated with Dowex chelating resin to remove trace metal ions and centrifuged (1788 *g*) and filtered to remove aggregates and resin. The concentration of contaminating copper in gamma globulin solutions made in this manner was 2 μM, as measured with a model 303 Perkin–Elmer atomic absorption spectrophotometer; the concentration of contaminating copper in the phosphate buffer used was 0.5 μM.

Bovine serum albumin (Fraction V powder for microbiological use) was obtained from Schwarz/Mann Research Laboratories, Orangeburg, NY. Human serum was obtained from healthy subjects, pooled, and stored at −20°. D-Penicillamine disulfide was used (Aldrich Chemical Co., Milwaukee, WI); all other amino acids were in the L form. Cupric sulfate was the source of added copper ion. Unless otherwise stated, concentrations of amino acids and metals refer to chemicals added and do not include contaminating metals or the small endogenous concentrations of L-histidine (10 μM) and copper (2 μM) present in the diluted human serum that was used [12].

Human gamma globulin. Human gamma globulin (2.5 mg/ml) was dissolved in 0.1 M phosphate–0.15 N NaCl buffer, pH 7.4. To denature the gamma globulin, 0.1-ml aliquots were heated to 90° for 10 min. After denaturation, 0.5 ml acetic acid was added (final concentration, 83.3%); the mixtures were centrifuged at 184 *g* for 10 min, washed three times, and assayed for protein as previously described [13]. Copper sulfate (0–10 μM) does not inhibit this reaction [12]. Control determinations (B in the legend of Fig. 1) were performed in triplicate with each run. Experiments were performed six times and the results averaged.

Bovine serum albumin and diluted human serum. Denaturation of bovine serum albumin (4.4 mg/ml) and diluted (1:10) human serum was carried out in 0.1 M chloride-free phosphate buffer, pH 7.4, at 75° for 2 hr. Each experiment was performed in triplicate. Viscosity measurements were made, also in triplicate, with an Otswald viscometer at 30° using 4 ml of a solution that had been centrifuged at 825 *g* for 5 min to remove occasional interfering particles. The transit time of phosphate buffer was 84.6 sec; the transit time of a solution of unheated bovine serum albumin was 85.6 sec; the transit time of unheated diluted human serum was 88.0 sec. Viscosity increment was defined as $(t/t_0) - 1$; t = transit time of heated test protein solution containing chemical reagent being studied; t_0 = transit time of heated control protein solution

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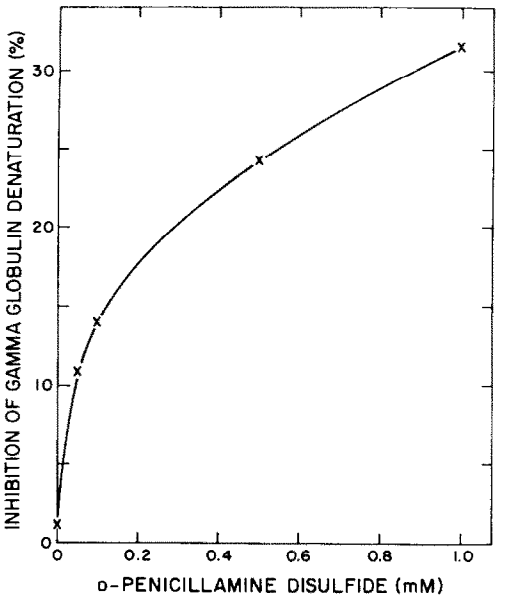

Fig. 1. Inhibition of the formation of acetic acid-insoluble heat-denatured human gamma globulin by D-penicillamine disulfide plus copper (2 μM $CuSO_4$ plus approximately 2 μM contaminating copper ion). Ordinate = 100(B-A)/B; A = acetic acid-insoluble gamma globulin formed in the presence of copper and varying concentrations of D-penicillamine disulfide, B = acetic acid-insoluble gamma globulin formed in the absence of D-penicillamine disulfide plus copper. B = 0.15 mg (S. E. = 0.007). The mean standard error of the inhibition of gamma globulin denaturation was 5.0 per cent (range 2.4 to 8.0). Inhibition was statistically significant ($P < 0.01$) at all concentrations of D-penicillamine disulfide used.

containing no test reagent; for bovine serum albumin, t_0 = 87.6 sec; for diluted serum, t_0 = 99.0 sec.

RESULTS

Human gamma globulin. Mixtures of D-penicillamine disulfide (50 μM to 1 mM) and copper sulfate (2 μM) inhibited the formation of acetic acid-insoluble denatured human gamma globulin by heat (Fig. 1). The inhibitory effect of 1 mM D-penicillamine disulfide plus 2 μM $CuSO_4$ was not suppressed by 25 μM histidine but was completely suppressed by 100 μM histidine. Copper ion was an essential component of the reaction mixture. D-Penicillamine disulfide (1 mM) in the presence of 50 μM EDTA was inactive. The requirement for copper in the D-penicillamine disulfide–copper mixture was specific: 2 μM Ca^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Na^+, Zn^{2+} and SO_4^{2-} were ineffective. Alanine, arginine, aspartic acid, glutamic acid, glycine, leucine, phenylalanine, proline, serine, threonine, tyrosine and uric acid (all 1 mM) were ineffective as substitutes for D-penicillamine disulfide in the D-penicillamine disulfide–Cu(II) mixture. D-Penicillamine disulfide plus Cu^{2+} had no effect on precipitate size when added after denaturation.

Bovine serum albumin and diluted human serum. An increase in viscosity occurred when solutions of bovine serum albumin (Fig. 2) and diluted human serum (Fig. 3) were thermally denatured in the presence of 20 μM Cu^{2+} plus varying concentrations of D-penicillamine disulfide. The addition of 4 mM histidine hardly changed the inhibitory effect of D-penicillamine disulfide plus copper (Figs. 2 and 3). Copper(II) alone was not significantly inhibitory ($P > 0.05$). In the presence of 10 mM EDTA, D-penicillamine disulfide and histidine, separately and together, had either less than or the same inhibitory effect as EDTA alone (Figs. 2 and 3).

D-Penicillamine disulfide (2 mM) plus 20 μM Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Na^+, Zn^{2+} or SO_4^{2-} produced no measurable inhibitory effect in either the bovine serum albumin or the diluted human serum system. D-Penicillamine disulfide (2 mM) plus Co^{2+} (20 μM) was 55 per cent (bovine albumin) and 33 per cent (serum) as inhibitory as D-penicillamine disulfide plus Cu^{2+}. D-Penicillamine disulfide plus Ni^{2+} (20 μM) was approximately as inhibitory (94 per cent in albumin and 109 per cent in serum) as D-penicillamine disulfide plus Cu^{2+}. Alanine, arginine, aspartic acid, glutamic acid, glycine, leucine, phenylalanine, proline, serine, threonine and tyrosine (all 2 mM) were inactive as substitutes for D-penicillamine disulfide in the D-penicillamine disulfide–copper(II) mixture. Sulfhydryl-inhibiting reagents produced viscosity increments similar to those of mixtures of D-penicillamine disulfide and Cu^{2+}. The viscosity increments produced by 0.2 mM *p*-chloromercuribenzoic acid in bovine serum albumin and diluted human serum were 0.183 and 0.540, respectively; for 0.2 mM *N*-ethylmaleimide they were 0.151 and 0.365, and for 0.2 mM gold thiomalate they were 0.080 and 0.192.

D-Penicillamine disulfide plus Cu^{2+}, with or without histidine, and EDTA, with or without histidine,

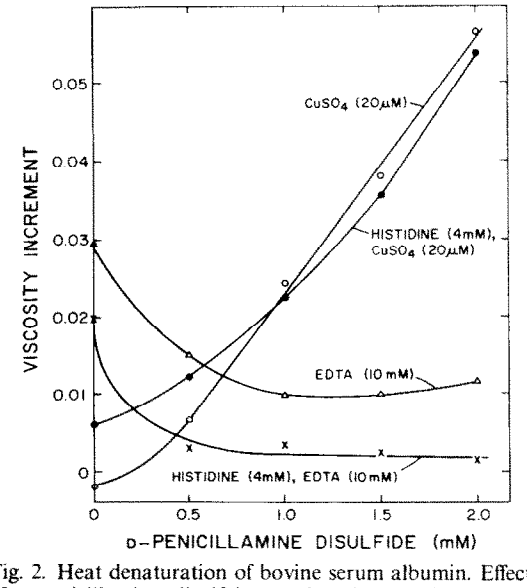

Fig. 2. Heat denaturation of bovine serum albumin. Effect of D-penicillamine disulfide on the viscosity increments produced by copper sulfate (○), L-histidine plus copper sulfate (●), EDTA (△) and L-histidine plus EDTA (×). Mean standard error of viscosity increments = 0.005 (range 0.001 to 0.013). The effect of D-penicillamine disulfide plus copper was statistically significant ($P < 0.05$) at D-penicillamine disulfide concentrations of 1.5 and 2.0 mM. The effect of D-penicillamine disulfide plus copper plus L-histidine was statistically significant ($P < 0.05$) at concentrations of D-penicillamine disulfide of 0.5 to 2.0 mM.

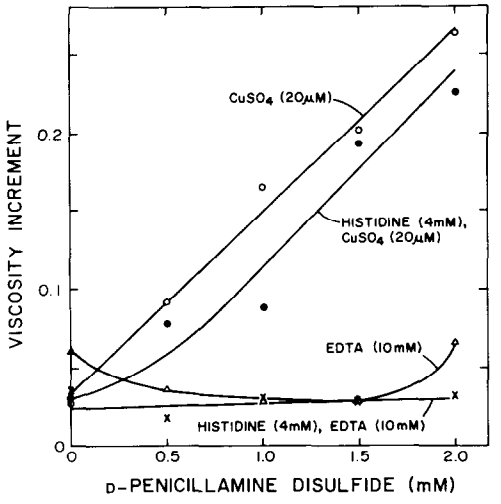

Fig. 3. Heat denaturation of diluted human serum. Effect of D-penicillamine disulfide on the viscosity increments produced by copper sulfate (○), L-histidine plus copper sulfate (●), EDTA (△) and L-histidine plus EDTA (×). Mean standard error of viscosity increments = 0.015 (range = 0.002 to 0.032). The inhibitory effect of D-penicillamine disulfide plus copper, with or without L-histidine, was statistically significant ($P < 0.05$) at D-penicillamine disulfide concentrations of 1.0, 1.5 and 2.0 mM.

had no effect on the viscosity of solutions of bovine serum albumin and diluted human serum prior to heat denaturation or when added after heat denaturation.

DISCUSSION

This study demonstrates that mixtures of D-penicillamine disulfide and copper(II) inhibit the thermal denaturation of human gamma globulin. A similar inhibitory effect has been demonstrated in the same system with three commonly used sulfhydryl-blocking reagents—*N*-ethylmaleimide, *p*-chloromercuribenzoic acid and iodoacetamide [12]. The denaturation-inhibiting effect of D-penicillamine disulfide–copper(II) mixtures was also shown with bovine serum albumin and diluted human serum. Two analytical methods were used—the measurement of acetic acid-insoluble aggregates and the measurement of viscosity. Both of these methods are thought to monitor the sulfhydryl-disulfide interchange reaction between protein molecules [12–19]. D-Penicillamine disulfide–copper(II) mixtures could inhibit sulfhydryl-dependent denaturation in two possible ways: (1) D-penicillamine disulfide could undergo a copper-catalyzed sulfhydryl-disulfide interchange reaction with protein sulfhydryl groups, analogous to the copper-catalyzed formation of mixed disulfides between cystine and beta globulin [20], or (2) the D-penicillamine disulfide–copper(II) complex could inhibit sulfhydryl group reactivity directly, analogous to the effects of gold thiomalate [16] and chloroquine [17] on heat denaturation.

It has been suggested that the hypohistidinemia of rheumatoid arthritis is pathogenetic in this disease by permitting hyaluronate-augmented [19] sulfhydryl-dependent denaturation of synovial fluid gamma globulin [12] and that in normal subjects this denaturation is prevented by adequate concentrations of the L-histidine–L-cystine–copper(II) complex [12]. This complex is thought to contain much if not most of the nonceruloplasmin copper in human plasma [5, 21–25]. Using the appropriate stability constants in a model simulating human blood plasma and a COMICS computer program, it has been calculated that D-penicillamine disulfide can compete for the L-cystine in this complex [5, 25]. The copper in both the cystine–copper complex [26] and the D-penicillamine disulfide–copper complex [27] is in the Cu(II) state.

L-Cystine–copper(II) mixtures also inhibit the heat denaturation of human gamma globulin, bovine serum albumin, and diluted human serum [12]. The ability of D-penicillamine disulfide (i.e. tetramethyl-D-cystine) to substitute for L-cystine in these mixtures is not surprising considering the close chemical similarity between the two compounds. However, the denaturation-inhibiting activity of L-cystine–copper(II) mixtures is considerably enhanced by L-histidine [12], whereas L-histidine did not have this effect on D-penicillamine disulfide–copper(II) mixtures.

The blood level of nonceruloplasmin copper (about 2 μM [12]) is similar to copper concentrations that were found to be effective *in vitro* in this study. The concentrations of D-penicillamine and D-penicillamine disulfide in the blood of patients with rheumatoid arthritis treated with D-penicillamine are not known. However, in a study of five patients treated with D-penicillamine for rheumatoid arthritis, no free D-penicillamine could be detected in the urine, and 39 per cent of the metabolized D-penicillamine in the urine was in the form of penicillamine disulfide [10]. The urinary forms of penicillamine-chelated copper have not been identified [28].

The present study used thermal denaturation at 75° and 90° as the test system. The relationship of denaturation at these temperatures to the possibility of chemically similar denaturation at body temperature is suggested by the recent demonstration of sulfhydryl-dependent mechanical aggregation of human gamma globulin at 37° [29].

Altered autologous IgG is inflammatory and antigenic [30, 31]. Aggregated gamma globulin [32] and immune complexes consisting of gamma globulin and anti-gamma globulin [33] have been found in the joint fluid of patients with rheumatoid arthritis. Immune complexes in joint fluid are generally thought to cause the inflammation of rheumatoid arthritis [31]. The present study suggests that D-penicillamine may suppress manifestations of rheumatoid arthritis by contributing to the formation of D-penicillamine disulfide–copper(II) complexes capable of inhibiting hypothesized non-immunological alteration of synovial fluid gamma globulin, thereby possibly preventing the formation of immune complex. Since denatured gamma globulin and immune complex are antigenic stimuli to the formation of rheumatoid factors [30, 31], this hypothesis could also explain the gradual decrease in titer of rheumatoid factor associated with the administration of D-penicillamine to patients with rheumatoid arthritis [11, 34] and the gradual increase in titer associated with D-penicillamine withdrawal [34].

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