Effect of penicillamine on complement *in vitro* and *in vivo*

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SUMMARY   In most normal human sera the addition of penicillamine to a final concentration of $0 \cdot 2$ mmol/l and subsequent dialysis caused a slight reduction in serum haemolytic complement (CH_{50}). At 200 mmol/l, CH_{50} activity was no longer demonstrable. Even higher concentrations of penicillamine were needed to inhibit the ability of immunoglobulin to fix complement to preformed or forming immune complexes. This indicated that the reduction of CH_{50} observed in serum was due to an effect on the complement factors. *In vivo*, a dose of 240 mg penicillamine caused a slight transient reduction in CH_{50} in rabbit serum, while 1000 mg penicillamine had no effect on serum CH_{50} in patients with rheumatoid arthritis. In arthritis patients there was, however, some evidence for removal of complement deposits in synovial tissue during penicillamine treatment. Since it is theoretically possible that concentrations high enough to cause reduction of complement activity can be achieved locally in synovial tissue, the effect on complement may be one of the mechanisms by which penicillamine exerts its effect in rheumatoid arthritis.

The effect of penicillamine in rheumatoid arthritis (RA) is no longer believed to be due to its splitting effect on IgM rheumatoid factors (Jaffe, 1970). However, if we assume that immune complex formation with subsequent complement fixation and activation are pathogenetic factors in RA (Zvaifler, 1973), there are several other immunological mechanisms which may explain the activity of penicillamine in this disease. One is that it acts as a general immunosuppressive agent. There is some experimental and clinical support for this (Altman and Tobin, 1965; Hübner and Gengozian, 1965; Bluestone and Goldberg, 1973), though in other studies no immunosuppressive effect (Liyanage and Currey, 1972) or even an enhanced immune response has been found (Tobin and Altman, 1964; Herrlinger *et al.*, 1975; Mellbye, 1977).

A second explanation may be that penicillamine, by forming mixed disulphides with proteins (Jellum and Skrede, 1977), inhibits complement binding to immunoglobulins in immune complexes and/or subsequent complement activation. Other disulphide-reducing agents have been shown to inhibit complement fixation to IgG (Wiedermann *et al.*, 1963;

Schur and Christian, 1964). It is also known that complement activation depends on a series of enzymatic steps, and it seems possible that one or more of these might be blocked by sulphydryl group-containing agents forming mixed disulphides. Therefore, the purpose of the present investigation was to test the effect of penicillamine on complement *in vitro* and *in vivo*.

Materials and methods

PENICILLAMINE
For the *in vitro* studies and the animal experiments D-penicillamine and N-acetyl-DL-penicillamine were obtained from Sigma Chemical Company (St. Louis, Mo., USA). For treatment of patients, D-penicillamine was given as Cuprimine (MSD, Rahway, N.J., USA).

TITRATION OF HAEMOLYTIC COMPLEMENT ACTIVITY (CH_{50})
This was performed as described by Mayer (1967).

TREATMENT OF SERA IN VITRO
Human serum was obtained from healthy blood donors and laboratory personnel. The sera were either tested immediately or stored at $-70°C$ until testing. Treatment with penicillamine was performed

by adding one volume of penicillamine solution in veronal-buffered saline (VBS), pH 7·2, to one volume of serum and incubating the mixture at 37°C for 30 minutes. In the initial experiments the mixture was then dialysed for 3 hours at room temperature against iodoacetamide in 15% molar excess, followed by dialysis overnight at 4°C against VBS. In later experiments dialysis against iodoacetamide was omitted.

Treatment of the rabbit antiserum to sheep red cells (amboceptor) with penicillamine was performed as described for human sera, except that treatment with iodoacetamide was omitted and dialysis against VBS was omitted in some of the experiments.

TREATMENT OF IMMUNE COMPLEXES
Immune complexes, consisting of rabbit anti-BSA (bovine serum albumin) and its antigen, were produced at equivalence on the basis of a quantitative precipitation curve. The precipitates were washed three times and suspended in phosphate-buffered saline (PBS), pH 7·4. Titration experiments were performed to find the lowest amount of precipitates which gave a clear inhibition of CH_{50} in the test system used. This was done to obtain maximum sensitivity for detection of inhibition of the complement-fixing ability of the precipitates. The precipitate was added to a solution of penicillamine, and after 1 hour's incubation at 37°C, the precipitates were washed three times and resuspended in PBS.

TREATMENT OF RABBITS
Black and white land rabbits, weighing approximately 2·5 kg, were given intramuscular injections of penicillamine. Blood samples were obtained from marginal ear veins before the injection and at intervals afterwards.

TREATMENT OF PATIENTS
Patients with classical or definite RA, according to the criteria by the American Rheumatism Association (McEwen, 1972) were tested. To examine the effect on CH_{50} in serum, 1000 mg D-penicillamine was given orally in one dose to patients who had either not received the drug before or had been taken off the drug for at least 3 days. Blood was obtained immediately before administration and at intervals afterwards.

To test the effect on complement deposition in synovial tissue, specimens obtained by synovectomy were examined by immunofluorescence technique. Immediately after surgery the tissue was cut into small pieces, washed in PBS, embedded in OCT Tissue-Tec, and deep-frozen in dry ice/acetone before cutting in a cryostat. Frozen sections were stained with fluoresceinated rabbit antisera against

human immunoglobulins, complement factor C3, and fibrinogen as previously described (Munthe and Natvig, 1972). Tissue specimens were from symmetrical joints in 3 cases, both joints being at approximately the same clinical and radiological stages. In all joints the arthritis was active and of long duration (more than 6 months), and in all cases there was a clear indication for synovectomy. This was performed on one side just before the patient started treatment with penicillamine (up to a maximum of 750 mg daily after 3 months), and synovectomy on the contralateral joint was usually performed after 3 months' treatment. In some cases tissues were examined only after treatment.

QUANTITATION OF C3 AND C4, AND DETECTION OF C3 CONVERSION PRODUCTS
Quantitation was performed by radial diffusion in gel analysis (Mancini et al., 1965), and conversion of C3 was tested for by crossed immunoelectrophoresis against anti-C3 (Laurell, 1965).

Results

EFFECT ON COMPLEMENT FIXATION TO IMMUNE COMPLEXES IN VITRO
To test the effect of penicillamine on complement fixation to Ig before or during immune complex formation, rabbit antiserum to sheep red cells was treated with penicillamine before sensitization of sheep red cells for CH_{50} testing. In two experiments the treated antiserum was dialysed against VBS to remove excess penicillamine before sensitization, while in three experiments this was omitted to avoid a possible reversal of the effect before the interaction with the antigen on the sheep red cells. In all cases an inhibiting effect of penicillamine on complement fixation was observed only when the concentration was as high as 200 mmol/l. Two examples are shown in Fig. 1.

To test the effect on preformed immune complexes, precipitates made from rabbit antiserum to BSA and its antigen were used. After incubation with varying concentrations of penicillamine, the ability of the precipitates to fix complement, and thereby reduce CH_{50} in normal human sera, was neutralized in one serum when the concentration was as high as 20 mmol/l. In three other sera no significant effect was seen even when the concentration of penicillamine was increased to 200 mmol/l. Two examples are included in Fig. 1.

EFFECT ON HAEMOLYTIC COMPLEMENT ACTIVITY IN SERUM IN VITRO
When human sera were incubated with varying concentrations of penicillamine, there was a constant

dose-dependent decrease of CH_{50} in the reaction mixture. In five of the six sera tested there was a reduction of at least 15% in CH_{50} when the final
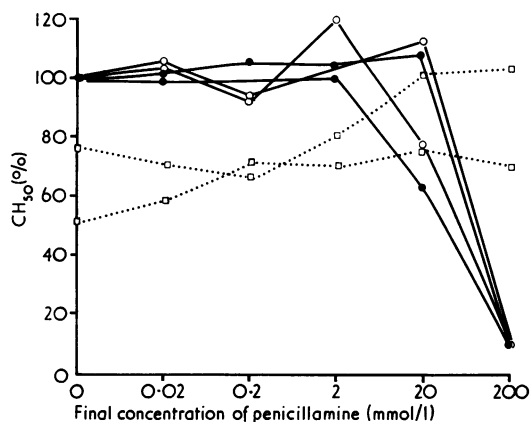

Fig. 1 *Effect of penicillamine on ability of immune complexes to fix complement* in vitro. *Solid lines show CH_{50} testing of normal human sera by sheep red cells sensitized with rabbit antiserum treated with D-penicillamine and either dialysed (●) or not dialysed (o) after treatment. Dotted lines show CH_{50} in sera after addition of BSA-anti-BSA (bovine serum albumin) precipitates treated with penicillamine. CH_{50} values are given as percentage of value with no penicillamine or precipitates added. Values below the lower limit of detection in the test system, 20%, are plotted as 10%.*
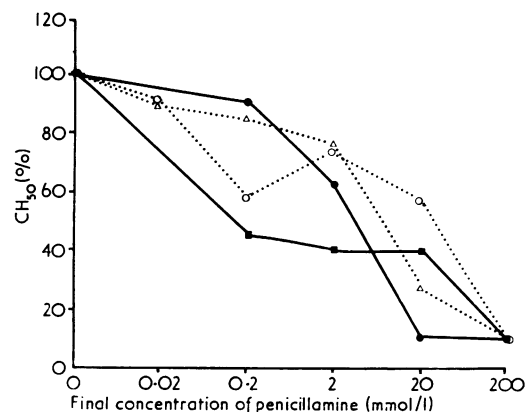
concentration of penicillamine was 0·2 mmol/l. At a concentration of 200 mmol/l the activity was in all cases below the lower limit of detection in our test system, corresponding to a decrease of at least 80%.

As shown in Fig. 2, there was no significant difference between the inhibiting effect of D-penicillamine and DL-acetyl-penicillamine. In these experiments the reaction mixture was treated with iodoacetamide after penicillamine to avoid possible reoxidation of reduced sulphydryl groups. However, as also shown in Fig. 2, there seemed to be no difference between sera treated with iodoacetamide and sera dialysed against PBS only.

The concentration of C3 and C4 were tested in four sera before and after treatment with D-penicillamine in a concentration of 200 mmol/l. No significant change was observed. Nor was any conversion of C3 found by crossed immunoelectrophoresis.

EFFECT ON RABBIT SERUM COMPLEMENT ACTIVITY IN VIVO

240 mg D-penicillamine was given intramuscularly to 5 rabbits and serum CH_{50} tested at intervals afterwards. In 2 rabbits the experiment was repeated after 5 days. The results of the seven experiments (Fig. 3) showed a great variation, but at 4 and 6 hours after administration, complement activity was reduced in all cases. Control experiments in 2 rabbits with injection of PBS alone indicated that the reduced activity seen after penicillamine was not due to the injection itself or to physiological diurnal variation in the animals.
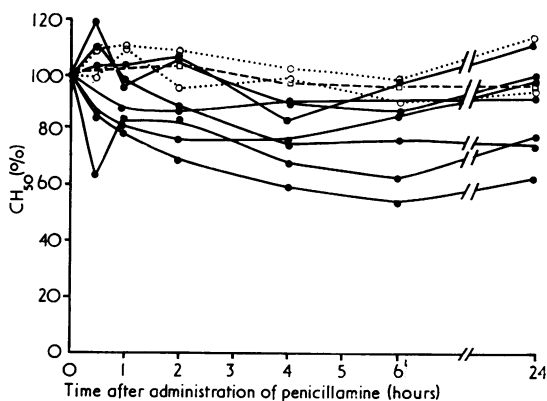

Fig. 2 *CH_{50} in serum after addition of penicillamine in vitro. Solid lines show CH_{50} in one serum treated with D-penicillamine (●) or DL-penicillamine (■), and dialysed against veronal-buffered saline (VBS). Dotted lines show CH_{50} in another serum treated with D-penicillamine and either treated with iodoacetamide (△) or not treated (o) before dialysis against VBS. Otherwise as for Fig. 1.*

Fig. 3 *CH_{50} in serum after in vivo administration of D-penicillamine. Solid lines show the results in rabbits; broken line the result in 1 patient with RA; dotted lines the results in control rabbits given phosphate-buffered saline only.*

EFFECT ON HUMAN SERUM COMPLEMENT IN PATIENTS WITH RA

Four patients with RA, 2 of whom had not been treated with penicillamine and 2 who had been taken off the drug for 3 days, were given 1000 mg penicillamine orally in one dose. CH_{50} was tested just before the drug was given and at intervals afterwards. No significant reduction of CH_{50} was observed. On the contrary, in one of the patients who had not received the drug previously there was a slight increase in CH_{50}. One typical example is included in Fig. 3.

### EFFECT ON DEPOSITION OF COMPLEMENT IN RHEUMATOID SYNOVIAL TISSUE

Before treatment with penicillamine synovial tissues examined by immunofluorescence technique usually showed large amounts of granular and/or lumpy deposits of immunoglobulins, complement factor C3, and fibrin. When tissues removed after 3 months of penicillamine treatment were examined and compared with the specimens taken before treatment,
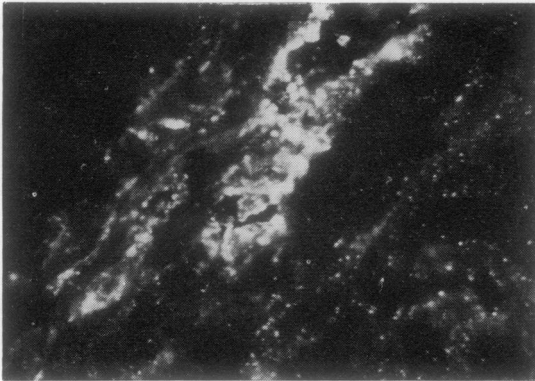
there appeared to be an overall, small reduction of the immunoglobulin content (Table). However, the amount of immunoglobulins varied considerably from one area to another in the same section.

One patient (Case 6) did not respond clinically to the treatment, and also had the largest amount of immunoglobulins in the tissues when examined after 6 months' treatment. Deposits of fibrin in the tissues showed no significant changes after the 3-month observation period. The only clear difference was a reduction in staining for C3 after treatment. The changes were particularly striking in Cases 1 and 3 (Table). As shown in Figs. 4a and b, the deposition of IgG was about the same before and after penicillamine, but the staining for C3 was clearly reduced (Figs. 5a, b).

(a)
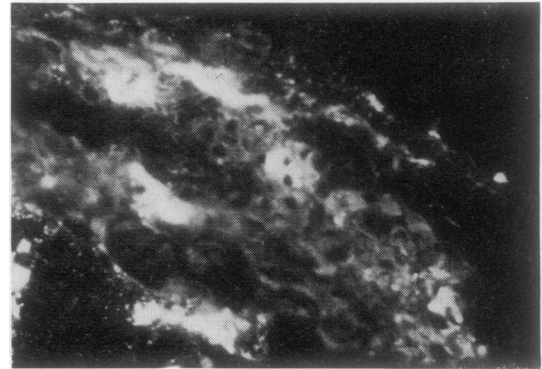
(b)

Fig. 4   (a) *Synovial membrane from Case 1 (left wrist) removed before penicillamine treatment and stained for IgG.* $\times 200$. *(b) Synovial membrane from right wrist of Case 1, removed after 3 months' penicillamine treatment and stained for IgG, showing no significant difference from (a).* $\times 200$.

Table   *Immunofluorescence microscopical observations before and after penicillamine treatment*

Case no.	Joint		F(ab')$_2$	IgG	IgM	IgA	C3	Fibrin
1	L wrist*	(before)	+++	++	+++	+	+++	+++
	R wrist	(after)	++	++	+++	+	(+)	+++
2	R wrist	(before)	+	++	(+)	0	+	++
	L wrist	(after)	+	+	0	0	0	++
3	R knee	(before)	++	+++	+++	++	+++	+++
	L knee	(after)	+	+	(+)	(+)	0	++
Only after treatment:								
4	R wrist		++	+	+	0	0	++
5	L flexor tendon sheath		+	+	(+)	0	0	+++
6	R knee		++	+++	+	++	+	+++

* Ig and C3 deposits in this issue were lumpy.
+++ =strong staining and large amounts; ++ =moderate staining; + =weak staining and small amounts.
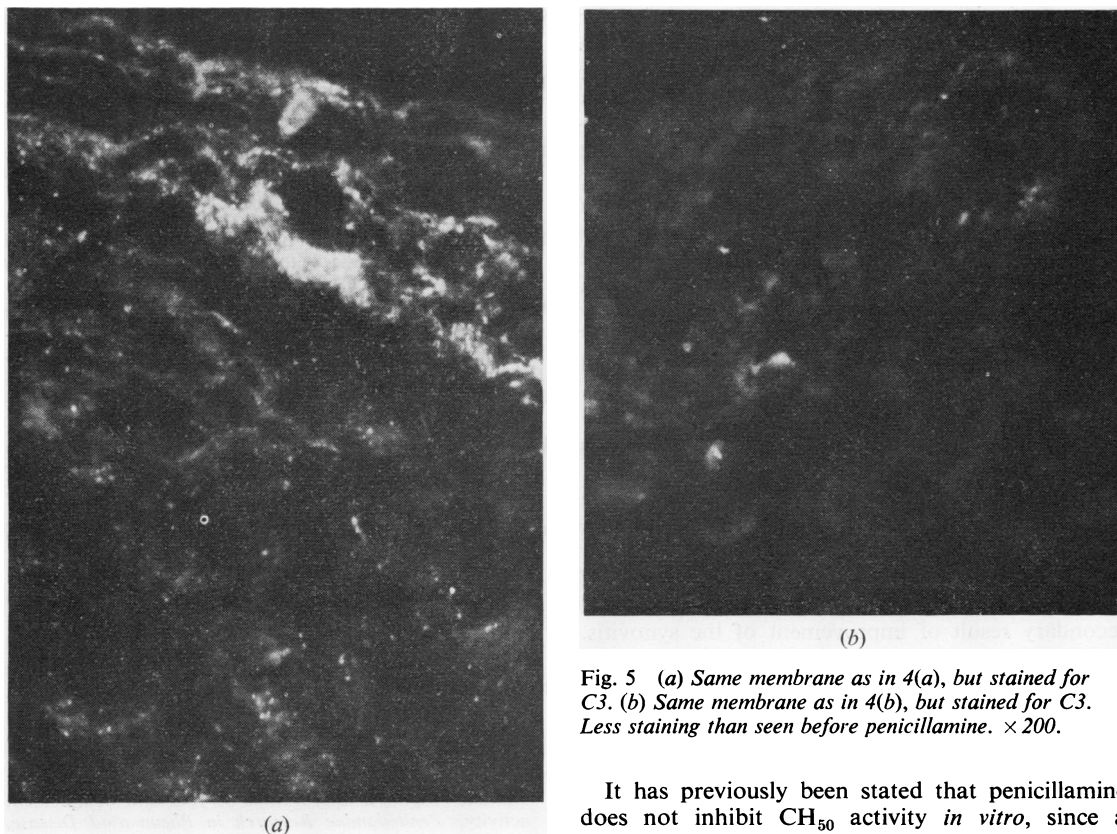Observation period for Cases 1, 2, 3 was 3 months.

(a)

(b)

Fig. 5   (a) *Same membrane as in 4(a), but stained for C3. (b) Same membrane as in 4(b), but stained for C3. Less staining than seen before penicillamine. × 200.*

Discussion

Our investigation showed that *in vitro*, penicillamine causes a dose-dependent inhibition of CH_{50} activity in serum. Using the same concentrations of penicillamine, there is little or no effect on the ability of immunoglobulins to fix complement to preformed or forming immune complexes. *In vivo*, a single relatively high dose of penicillamine caused a slight fall in serum complement activity in rabbits, but there was no similar effect in patients with RA. In RA patients there was, however, some evidence for removal of complement deposits in synovial tissue during penicillamine treatment.

It seems unlikely that the concentrations of penicillamine necessary to get a pronounced effect on complement *in vitro* is reached *in vivo* in blood in clinical situations. However, the concentration of penicillamine in synovial tissue is probably much higher than in serum (Ruiz-Torres, 1974), possibly as high as between 0·2 and 1·0 mmol/l (J. Aaseth, personal communication, 1976). Thus, the *in vitro* experiments may elucidate *in vivo* processes.

It has previously been stated that penicillamine does not inhibit CH_{50} activity *in vitro*, since a reduction of only 15 % was obtained with the highest concentration of penicillamine used, 2·7 mmol/l (Chwalińska-Sadowska and Baum, 1977). This result agrees with our own observations, and we had to increase the concentration to get a more pronounced inhibition.

The dose given in the rabbit *in vivo* experiments, approximately 80 mg/kg, may seem high when compared with the usual dose given to RA patients, usually up to 20 mg/kg. However, since the evaluation of the effect of drugs in various species should take into consideration the difference in metabolic rate (Berenbaum, 1975), these two dosages should be comparable.

Even if the concentration of penicillamine *in vivo* is high enough to inactivate complement, this does not necessarily mean that it has a beneficial effect on rheumatoid inflammation. However, there are experimental animal models which resemble RA in man and where decomplementation has been shown to prevent the development of the disease process (Cochrane, 1968).

It may be argued that since penicillamine is a chelating agent, able to bind divalent ions, the observed effect on CH_{50} activity may be due to

removal of calcium and magnesium ions needed for complement activity. This can be excluded since almost all samples were dialysed against buffer containing optimal concentrations of these ions before testing for CH_{50}. The possibliity that the effect of penicillamine was due to a splitting effect on the antiserum to sheep red cells, the amboceptor, which might have interfered with the test system for CH_{50} activity, was also excluded.

It is theoretically possible that the observed effect of penicillamine on CH_{50} is not due to an effect on the complement factors themselves but is caused by formation of complement-binding complexes of unknown composition. The finding of unchanged concentrations of C3 and C4, measured as protein antigens, and lack of C3 conversion products after incubation with penicillamine argues against this possibility.

The reduction in C3 deposition in synovial tissue after penicillamine treatment in patients with RA may be due to a direct effect of penicillamine on complement or the complement-binding ability of complexes in the tissue, though it may only be a secondary result of improvement of the synovitis. Moreover, there are several methodological difficulties involved in comparing immunofluorescence results in tissues obtained at different times.

It remains to be shown that penicillamine has a direct inhibiting effect on complement in rheumatoid synovial tissue. Taking into consideration all the various *in vitro* and *in vivo* effects of penicillamine (Munthe, 1977), it is likely that an inhibition of complement may be only one of several mechanisms for the therapeutic effect of this drug in RA.

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