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EVIDENCE FOR IMMUNE COMPLEXES CONTAINING ANTIBODY TO THE PEPSIN SITE OF IgG IN RHEUMATOID SYNOVIAL FLUIDS

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SUMMARY

In seventy cases of rheumatoid arthritis, the synovial fluid to serum ratio for the titre of pepsin agglutinator, i.e. the antibody to the pepsin site of IgG, was studied. This was compared with the corresponding ratio for another antibody of the same immunoglobulin class and for other proteins. By this method there was evidence for local production of pepsin agglutinator in two cases and for local inhibition in two other cases. Density gradient ultracentrifugation, performed at pH 7·4 and 3·0, suggested that pepsin agglutinator in immune complexes was present in most of the synovial fluids containing this antibody. Complexes of pepsin agglutinator and $F(ab')_2$ IgG, prepared *in vitro*, appeared to fix only small amounts of human complement. Neither did fixation of pepsin agglutinator to immune precipitates increase their complement binding activity.

INTRODUCTION

Besides rheumatoid factor (RF), sera and synovial fluids from patients with rheumatoid arthritis often contain other anti-γ-globulin antibodies (Kunkel & Tan, 1964). Among these are antibodies which react specifically with structures revealed by pepsin treatment of IgG (Osterland, Harboe & Kunkel, 1963) or IgA (Wilson, Soltis & Williams, 1970). The antibody to the pepsin site of IgG, later referred to as pepsin agglutinator (PA), also reacts with immune precipitates (Harboe, Rau & Aho, 1965; Mellbye & Natvig, 1970). Although PA is often present in sera from normal individuals, its frequency and activity is considerably higher in sera from patients with rheumatoid arthritis (Osterland *et al.*, 1963; Harboe *et al.*, 1965; Natvig, 1966; Kormeyer, Ing & Mandy, 1968).

The possible pathogenetic role of PA in rheumatoid arthritis is unknown. However, PA-producing cells have been demonstrated in synovial tissue of patients with rheumatoid arthritis (Munthe, 1969) and in eluates from such tissues (Munthe & Natvig, 1971).

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According to its specificity, PA in the eluates should represent antibody which has reacted *in vivo* with $F(ab')_2$ IgG or IgG in immune complexes. $F(ab')_2$ IgG may be produced in rheumatoid synovial fluid by lysosomal cathepsins (Fehr, LoSpalluto & Ziff, 1968). Quismorio, Rawson & Abelson (1968) reported that repeated intra-articular injection of Fab fragments into rabbit knee joints led to a chronic synovitis. PA might also be fixed *in vivo* to IgG in immune complexes since complexed IgG has been demonstrated both in synovial fluids (Hannestad, 1967; Winchester, Agnello & Kunkel, 1969) and in rheumatoid tissue (Kinsella, Baum & Ziff, 1969; Munthe, 1969).

Immune complexes are today considered as possible pathogenetic factors in the rheumatoid inflammation. The purpose of the present investigation was to study whether PA is specifically produced or inhibited in the joints of patients with rheumatoid arthritis, and if there is evidence for *in vivo* complex formation between PA and its antigen in synovial fluids from such patients. In addition, the complement binding activity of such complexes, prepared *in vitro*, was examined.

MATERIALS AND METHODS

Sera and synovial fluids were from patients with rheumatoid arthritis, hospitalized at Oslo Sanitetsforening Hospital for Rheumatic Diseases. Usually serum and synovial fluid from a patient were collected on the same day, or with an interval of up to 5 days. In a few cases the interval was up to 3 weeks. The material was stored at −20°C until tested. Before use the synovial fluids were treated with hyaluronidase as described by Hannestad & Mellbye (1967).

Phosphate buffered saline (PBS) was produced by adding 1 volume of a 0·1 M phosphate buffer at pH 7·4 to 9 volumes of saline.

Total protein concentration was measured by the Folin method, using a modification by Lowry *et al.* (1951).

Titration of PA and rheumatoid factor was performed by mixing 1 drop of doubling dilutions or serum fractions, with 1 drop of PBS and 1 drop of a 1% suspension of human group OR_1R_2 red cells sensitized with: either human IgG, using human anti-CD serum Ripley (kindly provided by Dr Marion Waller, Richmond, Virginia, U.S.A.) or pepsin treated human IgG, using the anti-CD Ripley antibody after pepsin treatment. The tests were performed on glass slides as described previously (Mellbye & Natvig, 1970).

In inhibition experiments 1 drop of serum dilution was mixed with 1 drop of the solution to be tested for inhibiting activity. After 5 min 1 drop of red cell suspension was added, and the procedure was continued as for the agglutination tests.

Titration of natural antibody to rabbit red cells was performed as for PA and rheumatoid factor, except that rabbit red cells were used instead of human red cells.

Pepsin treatment of IgG was performed as previously described (Natvig, 1970).

Density gradient ultracentrifugation was performed in a Spinco preparative ultracentrifuge type L50, using the technique of Fudenberg & Kunkel (1957). The sucrose solutions were either prepared in PBS or 0·1 M citrate buffer of pH 3·0. The concentrations ranged from 10 to 40%. Before centrifugation at pH 3·0, the samples were dialysed against the citrate buffer. Prior to the agglutination tests, the fractions were dialysed for 24 hr against PBS.

Splitting of disulphide bonds was performed with 2-mercapto-ethanol obtained from Sigma Chemical Company, St Louis, Missouri, U.S.A. Serum was diluted 1:2 in PBS and

2-mercapto-ethanol added to a final concentration of 0·2 M. The mixture was placed at room temperature for 45 min and then tested for agglutinating activity.

IgG, IgM and C3 (β_1C/β_1A) were measured by radial diffusion in gel technique as described by Fahey & McKelvey (1964). Antisera to IgG and IgM were obtained by immunization of rabbits with IgG (Kabi, Stockholm, Sweden), or IgM isolated from serum of a patient with macroglobulinaemia Waldenström, respectively. For quantitation of C3, Hyland's Immuno-Plates (Mesa Costa, California, U.S.A.) were used.

Immune precipitates containing human antidiphtheria toxoid were kindly provided by Dr K. Aho, Helsingfors, Finland, and kept at −20°C until used.

Testing of complement fixation. As human complement source, a pool of fresh normal human sera (NHS), stored at −70°C and containing PA and RF in a titre of less than 16, was used. As guinea-pig complement was used a 5% dilution of guinea-pig serum stored at −20°C. The sera were absorbed with sheep red cells at 0°C before use. To 1 volume of these sera was added 1 volume of serum with a high or low concentration of PA and 1 volume of a solution of pepsin treated IgG or a suspension of immune precipitates containing human antidiphtheria toxoid 2mg/ml. As controls, PBS was added instead of the inactivated serum, the pepsin treated IgG, or the precipitates, respectively. After 30 min incubation the precipitates were spun down. The haemolytic complement activity in the supernatant was tested either by adding SRC sensitized with rabbit haemolytic antiserum (Wellcome Research Laboratories, Beckenham, England) and reading the haemolysis by inspection after 30 min incubation at 37°C, or by measuring the CH_{50} by use of the same sensitized cells as described by Mayer (1961).

EXPERIMENTS AND RESULTS

Synovial fluid to serum ratio for PA

Sera and synovial fluids from seventy patients with rheumatoid arthritis were tested for their content of PA. In sixty-one patients the antibody was present in serum or synovial fluid in a titre of 16 or higher, which was judged as positive. In fifty-seven of these cases the titre in synovial fluid was equal to, or up to two titration steps lower than in the corresponding serum, i.e. the synovial fluid to serum ratio for the titre of PA was between 1/1 and 1/4. From previous work on other antibodies, it is assumed that this range of ratios represents a systemic antibody production followed by a transfer from serum to synovial fluid (Hannestad & Mellbye, 1967).

In the four remaining patients, all with a typical rheumatoid arthritis, the synovial fluid to serum ratio for PA was 4/1 in two cases, in the other two 1/16. This is shown in Fig. 1, which, for comparison, also includes the corresponding ratio in five cases randomly chosen from the group with a ratio between 1/1 and 1/4. The five cases will later be referred to as the control group. The titres of PA in the cases with a high or low ratio and the cases in the control group are shown in Table 1.

To judge whether the difference between the ratios in the four cases and those of the control group was specific for PA, we compared the ratios with the corresponding ratio for another antibody of the same class.

The immunoglobulin class of PA was determined by density gradient ultracentrifugation. In all the sera and synovial fluids the PA activity was found corresponding to the lower part of the large protein peak, known to contain the IgG antibodies. For comparison we

therefore used the synovial fluid to serum ratio for the titre of the natural human IgG antibody to rabbit red cells. Although this antibody usually occurs as a mixture of IgG and IgM (Tönder & Milgrom, 1965), it was chosen since it is present in nearly all normal sera, and

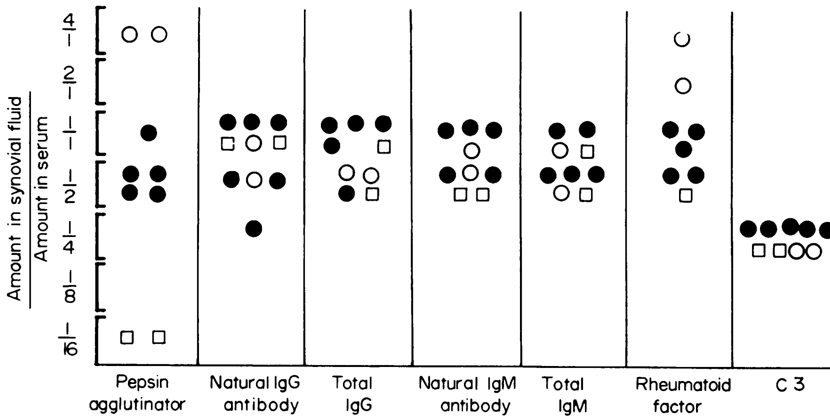

FIG. 1. Synovial fluid to serum ratio for the amount of pepsin agglutinator and other proteins in patients with rheumatoid arthritis. The results given are from cases with a high ratio for pepsin agglutinator (○), a low ratio (□), and from the control group (●). All ratios are expressed as proportions to facilitate comparison.

TABLE 1. Titres of pepsin agglutinator in synovial fluids and sera from patients with rheumatoid arthritis

		Synovial fluid	Serum
Cases with high synovial fluid to serum ratio for the titre of pepsin agglutinator	U.K.	256	64
	F.S.	64	16
Cases with low synovial fluid to serum ratio	K.L.	16	256
	S.O.	16	256
Control group	O.R.	128	128
	M.N.	128	128
	S.H.	64	128
	L.S.	128	256
	M.D.	32	64

since it is unlikely that it is specifically produced or inhibited in arthritic joints. To observe the effect of IgG antibodies only, the sera and synovial fluids were treated with 2-mercaptoethanol prior to the agglutination tests (Fudenberg & Kunkel, 1957).

As shown in Fig. 1, the four patients whose synovial fluids to serum ratio for PA differed from that of the control group, did not differ with respect to the corresponding ratios for

IgG antibody to rabbit red cells or total IgG concentration. The ratio for PA in the four patients was also judged to differ significantly from the ratios for the IgG antibody and total IgG in the same patients, since the difference was observed in repeated experiments. This indicated that in the two cases with the ratio of 4/1 for PA, this antibody had been produced wholly or in part in the joint, while in the two patients with a ratio of 1/16 PA had in some way been consumed or inhibited.

To exclude the possibility that the results were influenced by small amounts of IgM PA not detected by the density gradient ultracentrifugation, we also compared the ratio for PA with the corresponding ratio for an IgM antibody and total IgM globulin. As IgM antibody we used the so-called trypsin agglutinin, a natural IgM antibody to trypsin treated human red cells, present in all normal human sera (Mellbye, 1965). As shown in Fig. 1, the four patients whose ratio for PA differed from the control group, had a ratio for trypsin agglutinin and total IgM which was similar to that of the control group.

It has previously been shown that RF may both be produced and inhibited in the joints of patients with rheumatoid arthritis (Rodnan, Eisenbeis & Creighton, 1963; Bland & Clark, 1963; Hannestad & Mellbye, 1967). We therefore compared the synovial fluid to serum ratio for PA with the corresponding ratio for RF, as measured by titration against red cells sensitized with antiCD serum Ripley. The two patients with the high ratio for PA showed the same tendency in the ratio for RF. One of the patients with a low ratio for PA behaved as the controls in this respect, while the other was RF negative.

Since complement activity is frequently depressed in rheumatoid synovial fluids (Pekin & Zvaifler, 1962; Hedberg, 1963), we tested the synovial fluid to serum ratio for C3 as a positive control. In all the nine cases the ratio was 1/4, which, compared with the ratios for other proteins, was consistent with a specific consumption in the synovial fluid.

These experiments therefore indicated that among the seventy patients with rheumatoid arthritis, the results in two patients suggested a local specific production of PA in the synovial tissue, and in two other patients there was probably some sort of specific inhibition of PA in the joint.

Complexing of PA in joint fluids

When the joint fluids were tested by density gradient ultracentrifugation, the PA activity was in some cases found in somewhat deeper fractions than usually observed for IgG antibodies. This became still clearer when the same fractions were also tested for activity against rabbit red cells as a control. In five of eight synovial fluids the PA activity seemed to sediment faster than the IgG antirabbit red cell activity. In the corresponding sera, no significant similar discrepancy could be demonstrated. An example is included in Figs. 2(a) and 2(b).

The increased sedimentation rate of PA activity in these joint fluids might be due to a complex formation between this antibody and a specific inhibitor. To test this, the density gradient ultracentrifugation was repeated at pH 3·0, where antigen–antibody complexes are known to be dissociated. As shown in Fig. 2(a), the PA activity now sedimented as the IgG antirabbit red cell antibody. This phenomenon was observed in the synovial fluid from the two patients with the low synovial fluid to serum ratio for PA. It was also observed in synovial fluid from two of the controls, indicating that a certain complexing of PA in synovial fluids may occur more frequently than can be judged from the titration technique used initially. The phenomenon was not observed in the joint fluid of the two patients with the high ratio for PA or in any of the sera.

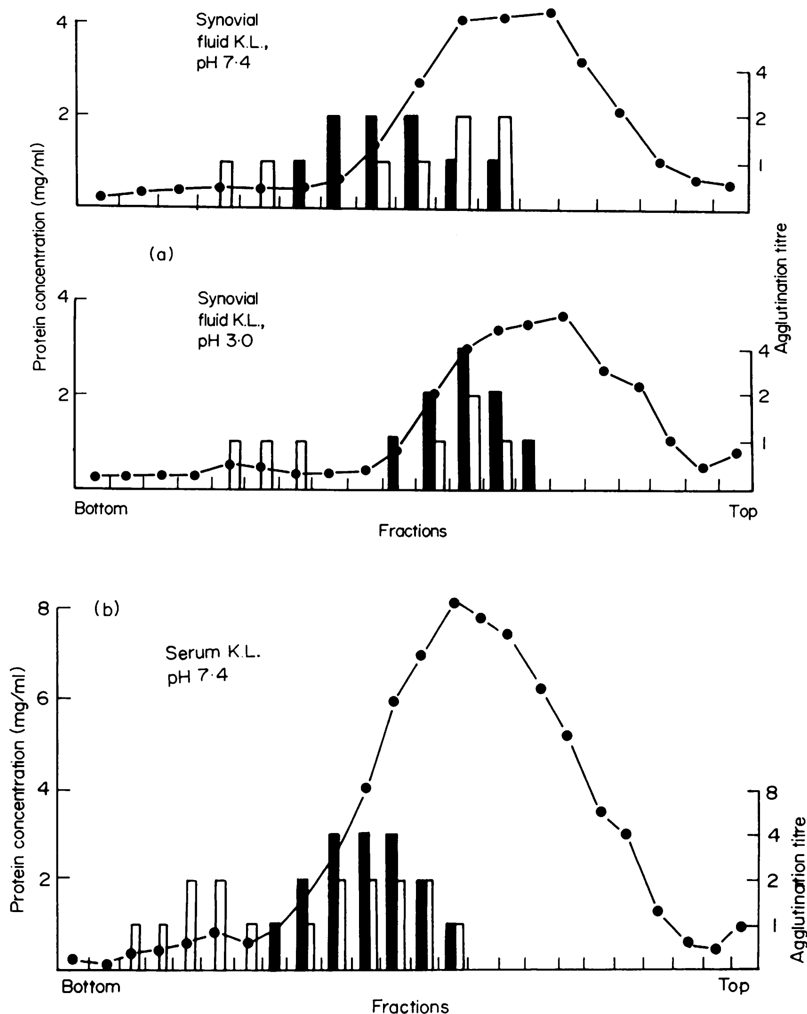

FIG. 2. Agglutinating activity of pepsin agglutinator (black columns) and natural antibody to rabbit red cells (open columns) in fractions obtained by density gradient ultracentrifugation of synovial fluid (Fig. 2a) and serum (Fig. 2b) from a patient with rheumatoid arthritis. The solid lines show the protein concentration.

To test the possibility that the increased sedimentation rate of PA was due to inhibition by $F(ab')_2$ IgG in the synovial fluids, we studied the effect of adding such fragments to three sera containing IgG PA. As shown in the example given in Fig. 3, by density gradient ultracentrifugation there was good correspondence between PA activity and IgG anti-rabbit red cell activity at pH 7·4 prior to the addition of $F(ab')_2$ IgG. After addition, in two of the sera some PA activity sedimented faster than the other IgG antibody at pH 7·4, while there was no difference when the centrifugation was performed at pH 3·0.

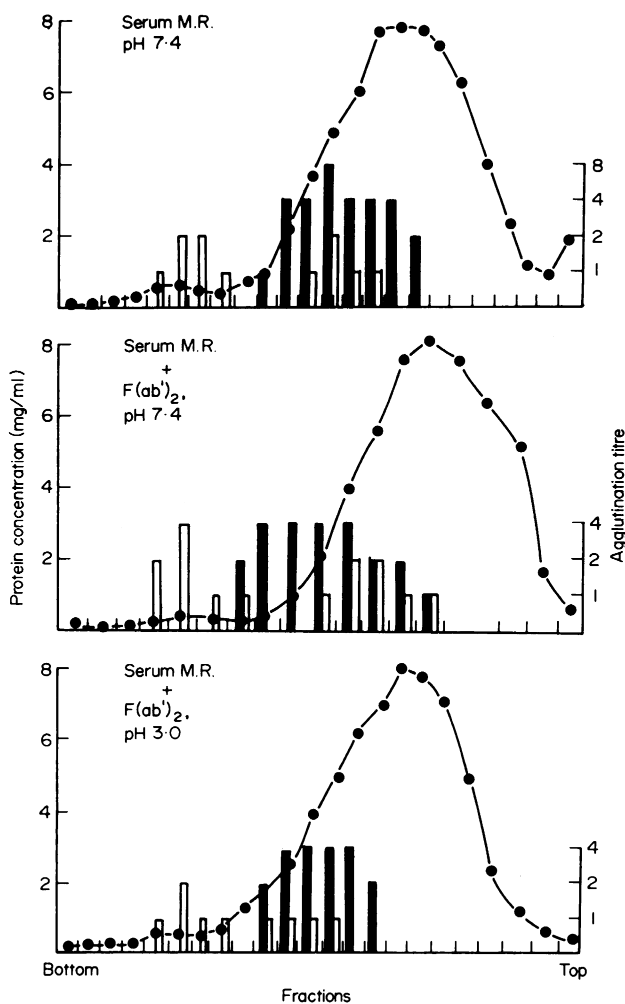

FIG. 3. Activity of pepsin agglutinator (black columns) and natural antibody to rabbit red cells (open columns) in fractions obtained by density gradient ultracentrifugation of serum from a patient with rheumatoid arthritis. The ultracentrifugation was performed before and after additon of $F(ab')_2$ IgG to a final concentration of 1 mg/ml and with different pH values in the sucrose gradient.

Complement fixation by complexes of PA and $F(ab')_2$ IgG

If PA fixed to $F(ab')_2$ IgG or immune complexes in arthritic joints is able to fix and activate complement, it might be involved in the release of the arthritic inflammation. We therefore tested the complement binding activity of twelve sera containing PA, where $F(ab')_2$ IgG had been added to a final concentration of 1 mg/ml. This addition completely inhibited the PA activity. Using 5% guinea-pig serum as complement source, and measuring the haemolytic activity after addition of sheep red cells sensitized with rabbit haemo-

lytic antibody, addition of $F(ab')_2$ IgG caused no significant reduction of the haemolytic complement activity.

Since human RF has been shown to fix human complement, but not guinea-pig complement (Zvaifler & Schur, 1968), the test was repeated in five sera, using fresh NHS, absorbed with sheep red blood cells, as complement source and measuring the haemolytic complement activity as the CH_{50} (Mayer, 1961). As shown in Table 2, addition of $F(ab')_2$ IgG to a final concentration of 1 mg/ml in all cases reduced the haemolytic complement activity in the reaction mixture. However, the reduction was usually not larger than that obtained by addition of $F(ab')_2$ IgG to NHS alone. Since the latter reduction might be due to a reaction between the small amount of PA in NHS and $F(ab')_2$ IgG, an additional control was performed with NHS absorbed with human red cells sensitized with pepsin treated antiCD Ripley antibody to remove PA activity. This reduced the complement fixation slightly, but not totally, indicating that some fixation of complement occurred through the $F(ab')_2$ fragments used.

TABLE 2. Haemolytic complement activity (CH_{50}) in mixtures of undiluted normal human serum (NHS), inactivated rheumatoid serum containing pepsin agglutinator (PA), and $F(ab')_2$ IgG

Fresh NHS + rheumatoid serum no.	Agglutination titre of PA in serum	CH_{50} in reaction mixture (% of CH_{50} in mixtures without $F(ab')_2$ IgG)
11646	128	63
11444	64	48
11121	128	76
13010	128	76
11646	64	90
Controls		
NHS + PBS	4	75
NHS (PA absorbed out) + PBS	0	91

In three of the sera, the test was repeated with $F(ab')_2$ IgG added to final concentrations ranging from 2 mg/ml to 0·1 mg/ml. There was still a weak reduction of the haemolytic complement activity, as measured by the CH_{50}. Controls demonstrated that the addition of $F(ab')_2$ IgG completely inhibited the PA activity in the sera, except for the lowest concentrations which only gave a partial inhibition.

Complement fixation by PA bound to immune precipitates

PA is known to react with immune precipitates containing human IgG as the antibody part (Harboe *et al.*, 1965; Mellbye & Natvig, 1970). We therefore tested the CH_{50} in mixtures of inactivated human sera with high (≥ 64) or low (≤ 4) titre of PA and immune complexes containing antidiphtheria toxoid, to which was added RF and PA negative fresh NHS as complement source. The amount of PA in the sera did not significantly influence the CH_{50} in the reaction mixture. Controls demonstrated that PA was fixed to the immune complexes during incubation.

DISCUSSION

By comparing the synovial fluid to serum ratio for the titre of PA with the corresponding ratio for another antibody of the same immunoglobulin class in seventy cases of rheumatoid arthritis, there was evidence for a specific local production of PA in two cases and a local inhibition in two cases. The results obtained by density gradient ultracentrifugation were consistent with presence of IgG PA in immune complexes in most of the synovial fluids. Complexes of PA and $F(ab')_2$ IgG, prepared *in vitro*, appeared to fix only small amounts of human complement. However, fixation of PA to immune precipitates made from human antidiphtheria toxoid did not increase their activity to fix complement. The reduction in CH_{50} was probably due to some fixation of complement factors to $F(ab')_2$ possibly in combination with PA. This might be related to the findings of Schur & Becker (1963).

All the synovial fluids and sera tested seemed to contain only IgG PA. This is in accord with most previous reports on this antibody, stating that PA is usually an IgG antibody (Osterland *et al.*, 1963; Harboe *et al.*, 1965; Natvig, 1970), but differs from the observations by Schoenfield & Epstein (1965), that PA in rheumatoid arthritis is most often an IgM antibody.

The present demonstration of local production of PA in arthritic joints resembles previous observations on RF (Hannestad & Mellbye, 1967). It is also consistent with the finding of Munthe (1969) that rheumatoid synovial tissue contains plasma cells producing PA. The antigenic stimulus for this production may be immune complexes, since such complexes have been shown to induce formation of PA in rabbits (Mellbye & Natvig, 1970). Complexed IgG has been demonstrated in rheumatoid synovial tissue (Kinsella *et al.*, 1969) and in eluates from the same tissue (Munthe & Natvig, 1971).

If the blocking or inhibition of PA observed in some of the joints is due to *in vivo* specific immunological inhibition, it should either be due to $F(ab')_2$ IgG or immune complexes. The inhibition might occur both in the synovial tissue and in the synovial fluid. As already mentioned immune complexes have been demonstrated in both these places and $F(ab')_2$-like fragments are possibly produced in rheumatoid synovial fluids (Fehr *et al.*, 1968).

The density gradient ultracentrifugation at neutral and lowered pH suggested that the inhibition of PA in synovial fluids was due to immune complex formation. The nature of the inhibiting factor is unknown, but the results obtained by adding $F(ab')_2$ IgG to sera *in vitro* suggests that this fragment may play a role also *in vivo*.

It may seem surprising that the presumed blocked PA was detected after density gradient ultracentrifugation. This may be due to removal of excess free reactants during centrifugation, thus making the PA available for detection in the agglutination tests.

The relation between the present possible inhibition of PA in rheumatoid synovial fluids and that previously observed in sera by Lawrence & Williams (1967) is not clear. We did not observe the increased sedimentation rate of PA during ultracentrifugation of sera, neither did we obtain significant inhibition of PA by use of PA negative fractions obtained by ultracentrifugation of synovial fluids or sera (Mellbye & Natvig, unpublished results). However, in pilot experiments with sera separated on G-200 Sephadex column, PA activity in some cases seemed to be eluted somewhat earlier than the bulk of IgG antibodies. This might indicate that our technique was relatively insensitive and detected only the stronger inhibitions taking place in the synovial fluids. A possible explanation for our observation of a more pronounced inhibition in synovial fluids than in sera, is that it is in both cases due

to a fragment of IgG which is predominantly produced and consumed in the joint. This would leave only small amounts for diffusion to serum and subsequent inhibition there.

Theoretically the changes observed in the present investigation might be due to inhibition of PA by fragments of IgG produced *in vitro*. The degradation of IgG during storage has been described by many authors (for references, see Art & Finlayson, 1969). The sera and synovial fluids used in the present experiments had been stored for up to 1½ years. However, they had been stored at −20°C, which should strongly reduce the degradation (Art & Finlayson, 1969). Moreover, we observed the PA inhibition phenomenon only in synovial fluids, although the sera and synovial fluids had been stored for the same time. Finally, one of the synovial fluids with a probable inhibition of PA had been stored for only 2 weeks prior to testing. It therefore seems unlikely that the present observations were due to *in vitro* alterations.

Immune complexes are believed to participate in the rheumatoid inflammation because of their ability to fix and activate complement. The present experiment indicated that the complex between PA and $F(ab')_2$ IgG fixed only small amounts of human complement. Similar observations have been made by Messner & Williams (1969), who could not restore the complement binding activity of pepsin-treated antibodies by adding PA.

The reason why PA seems to lack, or has a poor ability to fix complement, is not clear. It might be due to methodological factors, as the ability of an immune complex to fix complement depends on the antigen to antibody molecular ratio (Ishizaka, Ishizaka & Campbell, 1959). We tried to avoid this factor by using varying antigen to antibody ratios for testing, but this had no effect. Hypothetically, another explanation is that PA is restricted to the IgG4 subclass which fixes little or no complement (Müller-Eberhard, 1969).

Even if PA does not fix complement, immune complexes consisting of PA and $F(ab')_2$ IgG, or PA and other immune complexes might be of indirect pathogenetic importance by being phagocytosed by leucocytes which thereafter release their content of various enzymes.

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ABBREVIATION

PA pepsin agglutinator.