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Aggregation of Calcium Oxalate Crystals: Effect of Urine and Various Inhibitors

R. Felix, A. Monod, L. Broge, N. M. Hansen, and H. Fleisch

Department of Pathophysiology, University of Bern, Switzerland

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Summary. The influence of various factors on aggregation of calcium oxalate crystals in vitro was determined. Aggregation was assessed by filtering the crystal suspension and measuring the flow rate through a filter. 10% urine completely inhibited aggregation. Orthophosphate and magnesium at concentrations occurring in urine had no effect. Citrate had no effect at 10^{-4} M, but did inhibit at 10^{-3} M. The latter effect is probably due to calcium binding. Pyrophosphate and disodium dichloromethylene diphosphonate (Cl_2MDP) inhibited strongly at 10^{-4} M, disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) at 10^{-5} M, whereas pentanemonophosphonate had no effect. Uromucoid also did not show any inhibitory activity. Studies by means of heat inactivation, ultrafiltration and fractionation on DEAE-cellulose and gel-filtration indicated that the inhibitory activity was heterogenous and that the major part was larger than 10 000 daltons.

Key words: Calcium oxalate crystals - Aggregation - Urine - Pyrophosphate - Diphosphonates - Uromucoid.

A number of theories have been suggested to explain the process of urinary stone formation. Most emphasis has been placed on the role of supersaturation of the stone forming salts (15), on the role of the organic matrix (3) and on the roles of various inhibitors of crystal formation (5, 6, 13, 20). While these proposed mechanisms might be important in some of the patients, they are not the only explanation of the disease. Especially, they do not explain why many normal people have crystals in their urine but only some of them will form stones.

More recently it was reported that while normal people as well as stone formers excreted calcium oxalate crystals in their urine, the stone-forming patients tended to excrete large aggregates of crystals; this phenomenon was especially apparent after ingestion of oxalate (16, 17). It was found that urine inhibited the aggregation in vitro and it seemed that urine from patients with recurrent oxalate crystals produced less inhibition (17). It was therefore suggested that aggregation was important in urinary stone formation and that stone formers are less capable of inhibiting this process.

In view of the possible importance of the aggregation phenomenon in urolithiasis we have devised a new technique for measuring aggregation in vitro. We used this technique to investigate the inhibitory activity in urine and to study the role of various physiological compounds present in urine and of substances which might be used pharmacologically. Some of the results presented here were reported earlier at a symposium (9).

METHODS

Induction of Aggregation

The aggregation was produced in vitro by a procedure similar to that described by Robertson (17, 18). 250 mg of calcium oxalate monohydrate was added to 250 ml of a 0.15 M NaCl solution. This suspension was incubated at 37° C with magnetic stirring for 4 days or longer, in order to disperse the aggregates

initially present. The aggregation process was started by adding 200 µl of this suspension to 14 ml of a solution containing 5 mM sodium cacodylate, 1 mM $CaCl_2$, 0.2 mM sodium oxalate, 0.15 M NaCl, as well as urine or the various compounds to be tested. The solution was adjusted to pH 6.0 at 37° C, then incubated for 2 hours at 37° C in a plastic vial which was rotated at 500 rpm and then tested for aggregation. All solutions had to be free of particles and therefore had been filtered through Millipore filters (pore size 0.45 µm).

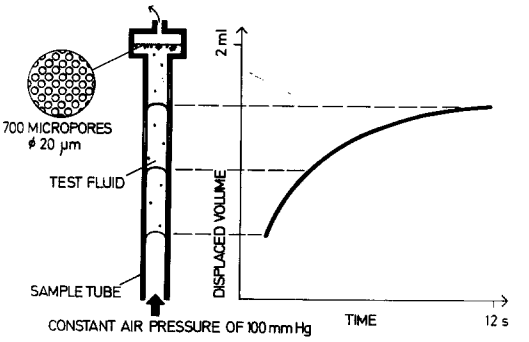

Fig. 1. Working principle of the agglometer

Measurements of Aggregation

An "Agglometer" (Institut Straumann, CH-4437 Waldenburg, Switzerland), recently devised to measure thrombocyte aggregation, was used. The technique consists of filtering the fluid containing the aggregates through a microporous filter, with a pore size of 20 µm, at a constant pressure of 100 mm Hg. The filtration was recorded graphically versus time (Fig. 1). With individual crystals of calcium oxalate that pass easily through the filter pores, the flow rate is constant and the record is a straight line. Aggregates larger than 20 µm do not pass the filter, so that it becomes progressively obstructed. Flow rate therefore diminishes with time and the record is a curved line. The amount of aggregation can thus be estimated by the height of the curve at an arbitrary time of 2 s, that is by the amount of fluid filtered at this time.

The results are expressed as the fluid volume filtered during 2 s. In some cases where the effect of urine was tested the aggregation was compared to a control aggregation and expressed as percentage of the control according to the following formula:

$$\% \text{ aggregation} = \frac{V_0 - V_u}{V_0 - V_c} = 100\,\%$$

V_0 = volume filtered in 2 s when a suspension was used before incubation

V_c = volume filtered in 2 s when a suspension was used which had been incubated in the absence of inhibitory compounds (control)

V_u = volume filtered in 2 s when a suspension was used which had been incubated in the presence of urine

Hydrolysis of Urinary Pyrophosphate

5 ml urine to which $(^{32}P)PP_i$ had been added and which had been adjusted to pH 6.0 were incubated with 10 units yeast inorganic pyrophosphatase at 25° C for 4 h. To check whether all the pyrophosphate had been hydrolysed an aliquot was taken after the incubation and orthophosphate and pyrophosphate were separated according to the method of Hall (12). Radioactivity was measured by Cerenkov counting in a Packard liquid scintillation counter.

Characterisation of Urinary Inhibitor

Fractionation According to Molecular Weight.

This was done by ultrafiltration through Amicon filters using UM-2 and UM-10 filters. The samples were rediluted with water to the original volume before the assay.

Fractionation with DE-22 and Gel Filtration.

1 l urine was concentrated on a UM-2 filter to 1/10, rediluted with distilled water to the original volume. It was then added to 75 g (dry weight) DE-22 cellulose which had previously been washed with 0.5 M HCl and 0.5 M NaOH and then been equilibrated with 10 mM acetate buffer, pH 4.0. After half an hour the slurry was centrifuged for 10 min at 3000 rpm, the supernatant decanted and assayed for activity (fraction I). The pellet was then successively extracted with 400 ml of the following solutions: 10 mM acetate at pH 4.0 (fraction II); 0.1 M piperazine-HCl at pH 6.0, 0.05 M NaCl (fraction III); four times with 0.1 M piperazine-HCl at pH 6.0, 1.0 M NaCl, giving fractions IV to VII. Each extract was tested for activity. The first extract with 1 M NaCl was then reconcentrated on an Amicon filter UM-2 and put on a Sephadex column G-75, 2.5 cm x 75.5 cm, which had been equilibrated with 0.1 M NaCl, 10 mM piperazine-HCl at pH 6.0. Cytochrome c was added as a marker protein. Fractions of about 3.7 ml were collected, so that 100 fractions corresponded to the total volume of the gel bed.

MATERIALS

Urine

Specimens were obtained from human males in the laboratory. They were filtered through filter paper and the pH adjusted to 6.0. If the whole urine or fractions of it had to be stored this was done at 4° C with addition of chloroform.

Uromucoid, uromucoid antiserum and urine from which the uromucoid had been removed by antibody precipitation were kindly supplied by Dr. K.H. Bichler, University of Marburg, W-Germany.

Chemicals

All chemicals were of analytical grade and obtained from Merck, W-Germany. The diphosphonates, disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP), disodium dichloromethylene diphosphonate (Cl_2MDP) and pentane monophosphonate (PMP), were from the Procter and Gamble Company, Cincinnati, Ohio, USA. DE-22 cellulose was obtained from Whatman, England; Sephadex G-75 from Pharmacia, Sweden; yeast inorganic pyrophosphatese from Boehringer, Mannheim, W-Germany, $(^{32}P)PP_i$ from New England Nuclear, Dreieichenhain, W-Germany.

RESULTS

Technique

The effect of 2 hours incubation is seen in Table 1. The decrease in the volume of fluid filtered reflects the increase in aggregation which occurred. The reproducibility is satisfactory.

Inhibitory Activity of Urine

As shown in Fig. 2 urine markedly retards the aggregation. Thus the addition of only 1% urine produces some inhibition while 10% blocks the process almost completely. It was also found that 5% urine, incubated with inorganic pyrophosphatase to destroy PP_i, does not reduce the inhibitory activity suggesting that PP_i does not represent a major part of the inhibitory activity.

The Effect of pH

As shown in Fig. 3 pH has only a small effect on the aggregation between 5.0 and 7.0, alkalinization retarding somewhat. The difference is significant for the control comparing pH 5.0 and 6.0 with pH 7.0 ($p < 0.01$). The pH does not influence the inhibition due to urine.

Table 1. Aggregation of calcium oxalate

Fluid filtered in 2 sec

	n	Volume (ml)	SE
No incubation	14	1.94	0.04
2 h incubation	16	0.82	0.03

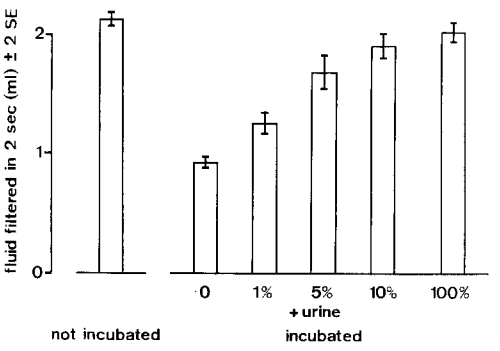

Fig. 2. Effect of urine on calcium oxalate aggregation

The Effect of Orthophosphate, Mg^{2+}, PP_i, Diphosphonates and a Monophosphonate

Orthophosphate (Fig. 4) has no effect between 10^{-4} and 10^{-2} M. Magnesium (Fig. 5) has no effect up to 10^{-3} M, but inhibits at 10^{-2} M. Citrate (not shown) does not inhibit up to 10^{-4}M, but does inhibit completely at 10^{-3} M. Pyrophosphate (Fig. 6) inhibits slightly at 10^{-5} M, strongly at 10^{-4} M. The diphosphonate EHDP (Fig. 7) starts inhibiting already at 10^{-6} M and blocks aggregation completely at 10^{-5} M. The diphosphonate Cl_2MDP (Fig. 8) had a pattern similar to pyrophosphate. The monophosphonate PMP (Fig. 9), however, has no effect. The effect of pyrophosphate and the diphosphonates is pH-dependent, being strongly activated by alkalinization (Fig. 10).

Effect of Uromucoid

As seen from Table 2, urine from which uromucoid had been removed by antibody precipitation did not differ from untreated urine. The control experiment, in which an equivalent amount of mucoid antiserum was

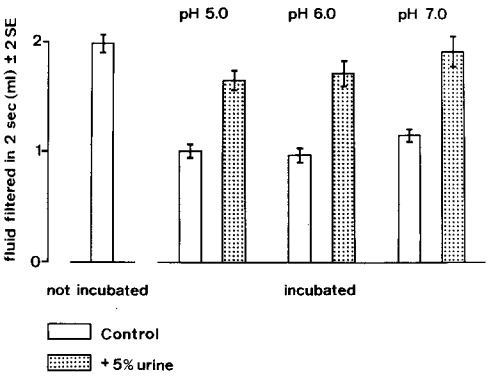

Fig. 3. Effect of pH on calcium oxalate aggregation

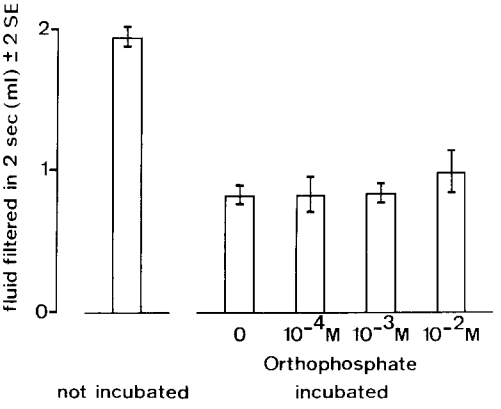

Fig. 4. Effect of orthophosphate on calcium oxalate aggregation

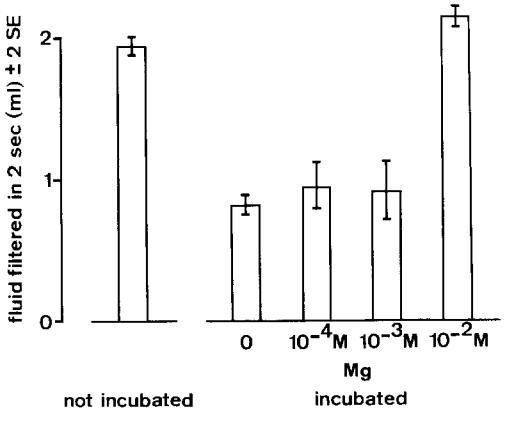

Fig. 5. Effect of magnesium on calcium oxalate aggregation

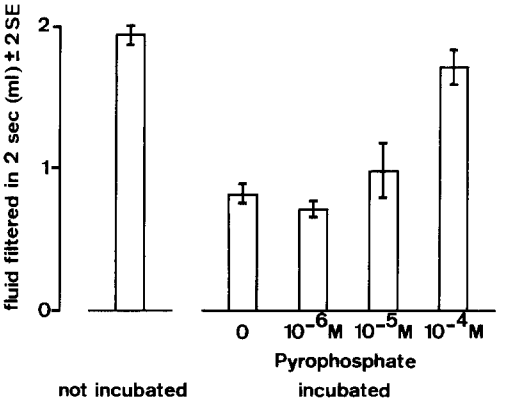

Fig. 6. Effect of pyrophosphate on calcium oxalate aggregation

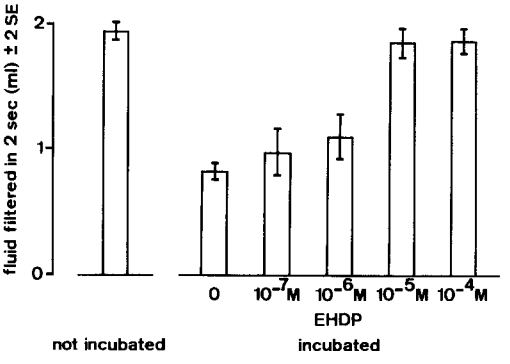

Fig. 7. Effect of ethane-1-hydroxy-1,1-diphosphonate (EHDP) on calcium oxalate aggregation

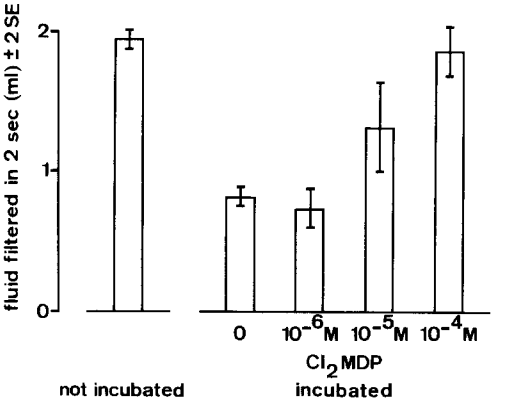

Fig. 8. Effect of dichloromethylene diphosphonate (Cl_2MDP) on calcium oxalate aggregation

added shows that the serum itself does not interfere with the assay. Table 3 shows that uromucoid when added to normal urine, to urine from which the uromucoid had been removed and to urine from a stone former in the concentration which would be present in 5% urine, also did not have any effect. In particular, the activity of the urine from one stone former was not increased. Finally increasing amounts of uromucoid up to an amount which would correspond to 40% urine, had no effect on aggregation.

Characterisation of Some Properties of the Urinary Inhibitors

As shown in Table 4 heating for 10 min in a boiling water bath did not destroy the inhibitory activity.

Table 5 shows the filterability of the in-

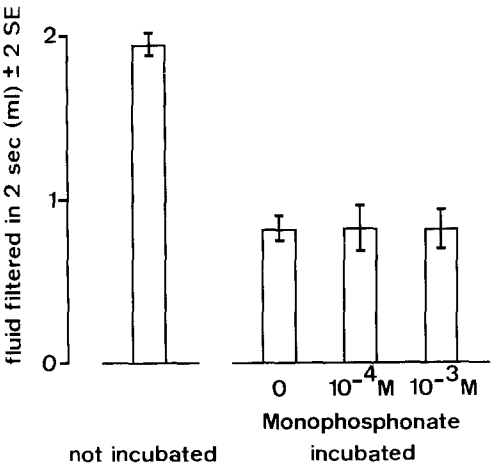

Fig. 9. Effect of n-pentanemonophosphonate (PMP) on calcium oxalate aggregation

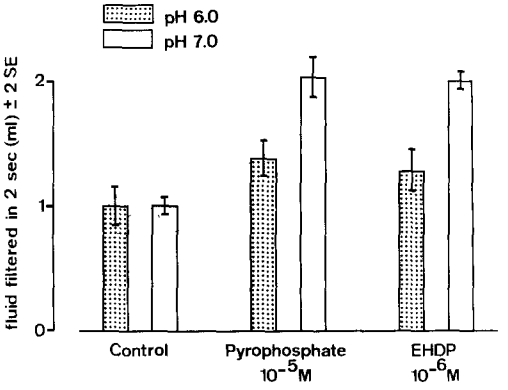

Fig. 10. Influence of pH on the inhibitory effect of pyrophosphate and ethane-1-hydroxy-1,1-diphosphonate

Table 2. Effect of uromucoid on calcium oxalate aggregation. Urine, urine without uromucoid and uromucoid antiserum were incubated with the calcium oxalate suspension at the given concentrations. 5% antiserum corresponds to addition of that volume of antiserum which would have been present in the aliquot of urine needed to make a sample solution containing 5% urine, provided that none of the antiserum was removed during the extraction procedure.

	Fluid filtered in two seconds (ml) mean ± S.E. (n)		
Concentration	Urine	Urine without uromucoid	Uromucoid antiserum
0 %	0.97 ± 0.04 (8)	0.97 ± 0.04 (8)	0.91 ± 0.04 (8)
1 %	1.27 ± 0.08 (6)	1.39 ± 0.11 (6)	
5 %	1.92 ± 0.04 (9)	1.84 ± 0.08 (9)	1.16 ± 0.06 (5)

Table 3. Effect of adding uromucoid to urine on aggregation of calcium oxalate aggregation

	Fluid (ml) filtered in 2 seconds (mean ± S.E.) at uromucoid concentration (= urinary equivalent added)			
	0 %	(n)	5 %	(n)
5 % Normal urine	1.86 ± 0.1	(3)	1.84 ± 0.05	(3)
5 % Normal urine without uromucoid	1.92 ± 0.09	(3)	1.73 ± 0.02	(3)
5 % "Stone-Former" urine	1.37 ± 0.06	(3)	1.49 ± 0.02	(3)

hibitory activity. Only a small part of the activity passed through an Amicon filter UM-2 with a cut-off of MW 1 000. A somewhat larger part passed through an UM-10 membrane with a 10 000 MW cut-off. Thus it appears that the activity is probably heterogenous, the largest part being, however, larger than 10 000 daltons.

As seen from Table 6, the inhibitors were bound to DE-22 cellulose equilibrated to pH 4.0. Raising the pH of the elution buffer to 6.0 did not wash out the inhibitors. By also increasing the NaCl concentration to 1.0 M the inhibitory activity was eluted, but not in one step. No inhibitory activity was eluted from the DE-22 cellulose itself by these procedures.

The first fraction eluted with 1 M NaCl from the DE-22 cellulose was reconcentrated on an UM-2 membrane and put then on a Sephadex G-75. Figure 11 shows that the inhibitory activity was eluted in two main peaks. The first seems to have a molecular weight between about 20 000 to 70 000, since it was eluted between the void volume (Mw = 70 000) and the cytochrome c (Mw = 13 000). A large part of the inhibitory activity, however, adhered to the resin and was eluted as a broad peak after the total volume.

Table 4. Stability of inhibitor to 10 min boiling. Results are expressed as aggregation ± S. E. in per cent of control aggregation without urine.

	% Aggregation (mean ± S. E.)	
Amount of urine	Not heated (n)	Heated (n)
1 %	49.7 ± 5.9 (6)	59.9 ± 8.7 (6)
5 %	18.9 ± 3.9 (6)	6.4 ± 5.6 (6)

DISCUSSION

Most studies have neglected the phenomenon of crystal aggregation in stone formation, despite the fact that some investigations (16, 17, 18) suggested that it might well be an important factor in stone formation. The aim of our studies was to define the factors influencing crystal aggregation.

Table 6. Purification of inhibitory activity. Results are expressed as aggregation as per cent of control aggregation. Aggregation inhibition of all fractions was determined at a concentration which corresponds to an equivalent of 5 % urine. (Time to filter 0.8 ml crystal suspension was measured instead of measuring the 2 second volume.)

	% Aggregation
Urine concentrated on an UM-2 and rediluted with water	16
Supernatant of DE-22 cellulose (fraction I)	125
Washing with 10 mM acetate, pH 4.0 (fraction II)	124
Washing with 0.1 M piperazine-HCl, pH 6.0 (fraction III)	118
Elution with 0.1 M piperazine-HCl, pH 6.0; 1 M NaCl:	
1. Elution (fraction IV)	40
2. Elution (fraction V)	39
3. Elution (fraction VI)	54
4. Elution (fraction VII)	60

Table 5. Filterability of the inhibitory activity. Results are expressed as aggregation ± S. E. in per cent of control aggregation without urine

	% Aggregation					
	UM-2 membrane			UM-10 membrane		
	Original urine (n)	Filtrate (n)	Rediluted concentrate (n)	Original urine (n)	Filtrate (n)	Rediluted concentrate (n)
1 % urine	33.8 ± 4.6 (6)	83.0 ± 5.5 (6)	36.5 ± 8.1 (6)	46.7 ± 4.7 (3)	68.5 ± 10.4 (3)	56.0 ± 8.9 (3)
5 % urine	8 ± 7.6 (6)	67.6 ± 6.9 (6)	5.7 ± 5.1 (6)	22.1 ± 11.4 (3)	48.6 ± 9.4 (3)	34.2 ± 5.9 (3)

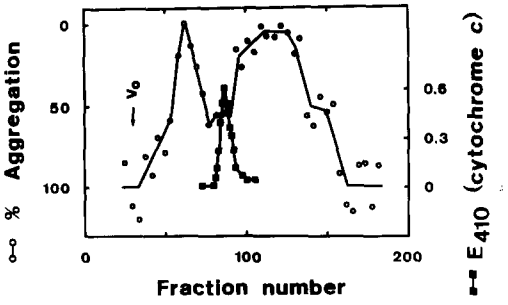

Fig. 11. Sephadex G-75 chromatography of urinary inhibitory activity. Aggregation is expressed as per cent of control aggregation and, as in Table 6, time was measured instead of the volumes

Urine had very powerful inhibitory activity, which is in accordance with other studies (17). Pyrophosphate, which is known to be a strong inhibitor of both calcium phosphate (4) and calcium oxalate (7) crystal formation, was not a major constituent of this inhibitory activity in the test system where 5% urine is used. However, as shown in Figure 6, PP_i between 10^{-5} M and 10^{-4} M, the concentration occurring in full urine, inhibited aggregation markedly. Thus the relative effect in normal urine remains uncertain until a test has been devised to measure aggregation in non-diluted urine.

pH had only a very weak effect, with or without the presence of urine (Fig. 3), so that it does not seem to be of importance to the activity of the inhibitors at these urinary concentrations. However, pH did have a strong effect on the action of pyrophosphate, inhibition being greater in an alkaline environment (Fig. 10). Thus the role of pH on the inhibitory activity of whole urine remains uncertain until the relative role of pyrophosphate in non-diluted urine can be determined.

Among the urinary constituents, neither orthophosphate, magnesium, nor citrate were effective. Probably the effect of high magnesium concentrations is due to complex formation between magnesium and oxalate, and of citrate due to complex formation between calcium and citrate. EDTA had an effect similar to that occurring with citrate.

Pyrophosphate, however, had a pronounced effect. The diphosphonates EHDP and Cl_2MDP, compounds related to PP_i but with a non-hydrolysable P-C-P bond, are known to inhibit calcium phosphate (8, 10) and calcium oxalate (11) crystallization in vitro and in vivo, and have therefore been suggested as useful agents in the treatment of urinary stones (1, 14, 19). These compounds had an even stronger effect on aggregation than pyrophosphate, which has also been found by other workers (18). The monophosphonate had no effect on aggregation.

Because of the heterogeneity the final characterization of the inhibitor, although of great clinical and physiological interest, is likely to be an arduous task.

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Dr. R. Felix
Pathophysiologisches Institut
der Universität Bern
Murtenstr. 35
CH-3010 Bern
Switzerland